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(54) Title: GROWTH AND DIFFERENTIATION OF STEM CELLS

(57) Abstract: The present invention relates to methods of culturing stem cells to produce hepatocyte-like cells. Among other advances, the invention also relates to purified preparations of hepatocyte-like cells and methods for using the hepatocyte-like cells.

## GROWTH AND DIFFERENTIATION OF STEM CELLS

### FIELD OF THE INVENTION

The present invention relates to methods of culturing stem cells to  
5 produce hepatocyte-like cells. The invention also relates to purified preparations  
of hepatocyte-like cells and methods for using hepatocyte-like cells.

### BACKGROUND OF THE INVENTION

The liver participates in many important physiological processes, including  
10 protein, lipid and carbohydrate metabolism, bile secretion, fibrinogen production  
and detoxification of a wide variety of foreign compounds and endogenous  
metabolites, including many therapeutic agents. By virtue of a portal circulatory  
system, the liver is the initial processing point for most materials absorbed  
through the gastrointestinal tract, and in this manner the liver protects the body  
15 from many harmful compounds. Hepatocytes are the most significant type of  
parenchymal cell in the liver, and hepatocytes perform most of the liver functions  
mentioned above.

In drug development, great significance is attached to the nature of the  
interaction between a candidate therapeutic and the cells of the liver. Many  
20 candidate therapeutics are significantly hepatotoxic. In addition, the  
pharmacokinetics of a candidate therapeutic are heavily influenced by the  
metabolic activities of hepatocytes. In vitro assays for predicting the in vivo  
hepatotoxicity and pharmacokinetics of a candidate compound are an important  
part of the drug development process.

25 Clinically, a ready source of metabolically active and transplantable liver  
cells would be invaluable. The liver is vulnerable to many disorders. A variety of  
compounds can cause temporary or permanent liver failure as well as liver cell  
death. In addition, a variety of diseases, such as hepatitis, may result in liver  
damage. While these conditions are usually correctable with a liver transplant,  
30 the chronic shortage of donor organs places this method of treatment out of  
reach for many patients.

Hepatocytes cultured from liver samples are useful for investigating the  
interaction between candidate therapeutics and the liver. However such cells  
are difficult to obtain in large numbers and cannot be maintained in culture as a

stable, genetically uniform cell line. In addition, primary hepatocytes have not been successfully used to restore liver function in a subject with a damaged liver, partly because they are difficult to obtain in sufficient quantities. For these and other reasons, it would be beneficial to have an alternate source for cells  
5 that have the characteristics of hepatocytes.

### SUMMARY OF THE INVENTION

The present invention relates generally to methods of culturing stem cells to produce hepatocyte-like cells. The invention also relates to purified  
10 preparations of hepatocyte-like cells and methods for using hepatocyte-like cells.

In a first embodiment, the invention relates to methods for obtaining a hepatocyte-like cell, comprising providing a stem cell, culturing the stem cell in a first medium comprising effective amounts of an acidic fibroblast growth factor (aFGF) and an epidermal growth factor (EGF) for about 2 to 4 days to obtain a  
15 first cell population, culturing a cell of the first cell population in a second medium comprising an effective amount of hepatocyte growth factor (HGF) for about 2 to 4 days to obtain a second cell population, and culturing a cell of the second cell population in a third medium comprising effective amounts of oncostatin-M for about 2 to 4 days to obtain a third cell population, the third cell population  
20 comprising a plurality of hepatocyte-like cells.

In a second embodiment, the invention relates to methods for obtaining a hepatocyte-like cell, comprising providing an ES cell, stimulating the differentiation of the ES cell into embryoid bodies for about 5 days, culturing the embryoid bodies in a first medium comprising effective amounts of an aFGF and  
25 an EGF for about 1 to 2 days to obtain embryoid bodies, dissociating the embryoid bodies into a single cell suspension and culturing the single cell suspension for about 1 to 2 days in the first medium to obtain a first cell population culturing a cell of the first cell population in a second medium comprising an effective amount of EGF, HGF and aFGF for about 2 to 4 days to  
30 obtain a second cell population, and culturing a cell of the second cell population in a third medium comprising effective amounts of oncostatin-M, EGF, and HGF for about 2 to 4 days to obtain a third cell population, the third cell population comprising a plurality of hepatocyte-like cells.

In a third embodiment, the invention relates to methods for obtaining a cellular composition comprising an enriched population of hepatocyte-like cells, comprising providing a cellular composition comprising a plurality of hepatocyte-like cells; and culturing the cellular composition in a medium suitable for  
5 selectively culturing gluconeogenic cells, thereby obtaining a cellular composition comprising an enriched population of hepatocyte-like cells.

In a fourth embodiment, the invention relates to cellular compositions comprising viable cells, wherein at least 90% of the viable cells are hepatocyte-like cells.

10 In a fifth embodiment, the invention relates to methods for determining whether a test agent is toxic to a hepatic cell, comprising contacting a hepatocyte-like cell according to the invention with the test agent for a time sufficient for any toxic effect on the cell to be detected, and determining the toxic effect on the cell.

15 In a sixth embodiment, the invention relates to methods for identifying a metabolic product of a test agent, comprising contacting a hepatocyte-like cell according to the invention with the test agent for a time sufficient for the test agent to be metabolized, and detecting the presence of a metabolized product.

20 In a seventh embodiment, the invention relates to methods for treating a subject in need of liver cells, comprising administering to the subject a therapeutically effective amount of the hepatocyte-like cells according to the invention.

In a eighth embodiment, the invention relates to isolated nucleic acids encoding a polypeptide having SEQ ID NO: 18.

25 In a ninth embodiment, the invention relates to isolated polypeptides comprising the amino acid sequence SEQ ID NO: 18.

In a tenth embodiment, the invention relates to methods for stimulating the proliferation of a hepatocyte-like cell or precursor thereof, comprising contacting a cellular composition according to the invention with an isolated  
30 polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 18.

### DETAILED DESCRIPTION OF THE INVENTION

The invention is based in part on the discovery that embryonic stem cells can be differentiated into a highly purified population of hepatocyte-like cells.

#### 1. Definitions

5 For convenience, certain terms employed in the specification, examples, and appended claims are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

10 The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "acidic fibroblast growth factor" or "aFGF" includes the native aFGF from any organism as well as any functional mimic thereof. An exemplary nucleotide sequence of human aFGF is set forth in SEQ ID NO: 1 and the  
15 protein encoded thereby is set forth in SEQ ID NO: 2, which sequences correspond to GenBank Accession Numbers NM\_000800 and NP\_000791.1, respectively. Other spliceforms of aFGF are set forth in GenBank Accession Numbers NM\_033137 and NM\_033136. An exemplary nucleotide sequence of a mouse aFGF is set forth in SEQ ID NO: 3 and the protein encoded thereby is set  
20 forth in SEQ ID NO: 4, which sequences correspond to GenBank Accession Numbers M30641 and AAA37618.1, respectively.

"Agent" refers to a chemical compound, such as small molecules and biological macromolecules (e.g., DNA, RNA, polypeptides or lipids).

25 "Cells" refers not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

30 The terms "cell culture" or "culture" include any combination of cells and medium. The cells need not be actively growing.

A "cellular composition" is a composition that comprises a plurality of viable cells, wherein the viable cells are not in their natural context. For example, cellular compositions do not include whole organisms. Cellular compositions may comprise materials (e.g. media, support matrix, dead cells,

pharmaceutically acceptable carriers etc.) in addition to the plurality of viable cells. Exemplary cellular compositions include liquid or plated cell cultures, cell suspension, cells seeded on a matrix, artificial tissue constructs, frozen cells, cells prepared with a suitable carrier for administration to a subject, etc. A viable  
5 cell is a cell that is capable of performing the metabolic functions of a cell when placed in the appropriate conditions. For example, a viable cell may be an actively growing cell, a cell no longer capable of undergoing cell division but nonetheless metabolically active or a frozen cell that is not metabolically active but may become metabolically active when thawed in the appropriate conditions.

10 A "cell population" is a plurality of cells.

An "embryonic stem cell" or "ES cell" refers to a totipotent stem cell isolated from the inner cell mass of an early stage blastocyst, as described, e.g., in E.J. Robertson "Embryo-derived stem cell lines, in Teratocarcinomas and embryonic stem cells: a practical approach, E.J. Robertson, editor, IRL Press,  
15 Washington D.C., 1987.

"Endogenous", in reference to a growth factor or other substance refers to the fact that the substance is in a form substantially similar to a form found in nature or a form predicted to be found in nature (i.e. a polypeptide including predicted glycosylation structures, even if such glycosylation structures have not  
20 been characterized in a form of the substance found in nature).

The term "epidermal growth factor" or "EGF" includes the native EGF from any organism, as well as functional mimics. An exemplary nucleotide sequence of human EGF is set forth in SEQ ID NO: 5 and the protein encoded thereby is set forth in SEQ ID NO: 6, which sequences correspond to GenBank Accession  
25 Numbers NM\_001963 and NP\_001954.1, respectively. An exemplary nucleotide sequence of a mouse EGF is set forth in SEQ ID NO: 7 and the protein encoded thereby is set forth in SEQ ID NO: 8, which sequences correspond to GenBank Accession Numbers J00380 and AAA37539.1, respectively.

"Fragment" as used in reference to a factor, e.g., a growth factor, includes  
30 polypeptides that have an amino-terminal and/or carboxy-terminal deletion relative to a naturally occurring form of the factor. An "active fragment" is a fragment that retains at least enough functional activity of the relevant factor that the active fragment may be used as a replacement for the factor. Optionally, an active fragment retains 25% or more of the activity of the relevant factor.

The term "gluconeogenic" used in reference to a cell refers to any cell that is capable of generating glucose from a simpler molecule than glucose, such as pyruvate, lactate, amino acids, glycerol or propionate. In mammals, gluconeogenic cells generally express some or all of the gluconeogenic enzymes: glucose-6-phosphatase, fructose-1,6-bisphosphatase, pyruvate carboxylase and pyruvate carboxykinase. In humans, only two cell types are known to be gluconeogenic: breast epithelium and hepatocytes.

A "hepatocyte-like cell" is a cell having a plurality, e.g., at least two, three, four, or five, of characteristics of a hepatocyte. Exemplary hepatocyte-like cells include cells having two or more of the following properties: the ability to use pyruvate as a sole carbon source; butyrate resistance at concentrations of at least about 1-20 mM and preferably at least about 5mM sodium butyric acid; the ability to take up vinblastine; cytochrome P450 activity (e.g., dibenzylfluorescein metabolism, dextromethorphan oxidation, coumarin glucuronidation or sulfation); cytochrome P450 expression (detected, for example, by RT-PCR or antibody staining); or expression of other genes and/or proteins that are indicative of hepatocytes, such as  $\alpha$ -fetoprotein,  $\gamma$ -glutaryltransferase, hepatocyte nuclear factor (HNF) 1 $\alpha$ , HNF 1 $\beta$ , HNF 3 $\alpha$ , HNF 3 $\beta$ , HNF 4, anti-trypsin, transthyretin, and CFTR. Hepatocyte-like cells may be immortalized or aneuploid, but need not be.

The term "hepatocyte growth factor" or "HGF" includes the native HGF from any organism, as well as functional mimics. An exemplary nucleotide sequence of human HGF is set forth in SEQ ID NO: 9 and the protein encoded thereby is set forth in SEQ ID NO: 10, which sequences correspond to GenBank Accession Numbers M29145 and AAA52650.1, respectively. An exemplary nucleotide sequence of a mouse HGF is set forth in SEQ ID NO: 11 and the protein encoded thereby is set forth in SEQ ID NO: 12, which sequences correspond to GenBank Accession Numbers D10212 and BAA01064.1, respectively.

The term "hepatopoietin" or "HPO" includes the native HPO from any organism, as well as functional mimics. Human HPO is a polypeptide mitogen that is described as consisting of a 15.1 kDa protein of 206 amino acids (see, e.g., Wang et al., J. Biol. Chem., 274:11469 (1999), Yang et al., Sci. China Ser. C Life Sci., 40:642 (1997), and GenBank Accession No. AF306863). Another

human HPO is encoded by the nucleotide sequence set forth in GenBank Accession number AF183892, encoding a protein of 180 amino acids having the sequence set forth in GenBank Accession number AAD56538. The human HPO is an orthologue of rat augmenter of liver regeneration or hepatic stimulator substance (see, e.g., Li et al. (2000) J. Biol. Chem. 275:37443). Rat HPO is a protein of 125 amino acids (see, e.g., Hagiya et al. (1994) PNAS 91:8142). An exemplary nucleotide sequence of a partial cDNA of human HPO is set forth in SEQ ID NO: 13 and the protein encoded thereby is set forth in SEQ ID NO: 14, which sequences correspond to GenBank Accession Numbers AF306863 and AAG38105, respectively. An exemplary partial nucleotide sequence of a mouse HPO is set forth in SEQ ID NO: 15 and the protein encoded thereby is set forth in SEQ ID NO: 16, which sequences correspond to GenBank Accession Numbers AF148688 and AAD36987, respectively. An exemplary full length nucleotide sequence of a mouse HPO is set forth as SEQ ID NO: 17 and the protein encoded thereby is set forth in SEQ ID NO: 18. A nucleic acid encoding a protein differing in one amino acid from SEQ ID NO: 18 (the residue at position 49 is an alanine instead of a serine) is set forth in GenBank Accession Number AB041561 and encodes the protein set forth in GenBank Accession Number BAA95045.

"Isolated" as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, an isolated nucleic acid encoding an HPO polypeptide includes no more than the entire gene (including the promoter), usually no more than 10 kilobases (kb) of nucleic acid sequence which naturally immediately flanks the HPO gene in genomic DNA, preferably no more than 5kb of such naturally occurring flanking sequences, and more preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated



from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

The term "medium", as used in reference to a cell culture, includes the components of the environment surrounding the cells. Media may be solid, liquid, gaseous or a mixture of phases and materials. Media include liquid growth media as well as liquid media that do not sustain cell growth. Media also include gelatinous media such as agar, agarose, gelatin and collagen matrices. Exemplary gaseous media include the gaseous phase that cells growing on a petri dish or other solid or semisolid support are exposed to. The term "medium" also refers to material that is intended for use in a cell culture, even if it has not yet been contacted with cells. For example, a nutrient rich liquid prepared for cell culture is a medium.

"Non-human animals" include mammals such as rodents, non-human primates, ovines, bovines, equines, porcines, canines, felines, chickens, amphibians, reptiles, etc.

"Nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

The term "oncostatin-M" or "OSM" includes the native oncostatin M from any organism, as well as functional mimics. An endogenous human oncostatin M is a 227 amino acid polypeptide with two glycosylation sites. An exemplary nucleotide sequence of human OSM is set forth in SEQ ID NO: 19 and the protein encoded thereby is set forth in SEQ ID NO: 20, which sequences correspond to GenBank Accession Numbers BC011589 and AAH11589.1, respectively. An exemplary nucleotide sequence of a mouse OSM is set forth in SEQ ID NO: 21 and the protein encoded thereby is set forth in SEQ ID NO: 22, which sequences correspond to GenBank Accession Numbers J04806 and AAA57265.1, respectively.

"Regulatory nucleic acid" means a DNA sequence that regulates expression of a selected DNA sequence operably linked thereto. Exemplary regulatory nucleic acids include promoters, enhancers, repressors, histone binding elements, etc.

The term "polypeptide", and the terms "protein" (if single chain) and "peptide" which are used interchangeably herein, refers to a polymer of amino acids. Polypeptides may also include one or more modifications such as, for example, a lipid moiety, a phosphate, a sugar moiety, etc.

- 5        "Recombinant protein" refers to a polypeptide that is produced by recombinant DNA techniques, wherein generally, DNA encoding a protein is inserted into a suitable expression vector which is in turn used to transform a cell to produce the protein.

10        The following terms are used to describe the sequence relationships between two or more polynucleotides or polypeptides: "reference sequence", "sequence identity", "percentage of sequence identity", and "substantial identity." A "reference sequence" is the sequence that forms the basis for comparison. In the phrase "a polypeptide comprising an amino acid sequence that is 95% identical to the amino acid sequence in SEQ ID NO:1", the reference sequence  
15        is the amino acid sequence shown in SEQ ID NO:1. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the length of the reference sequence. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the length of the reference sequence, determining the  
20        number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. Gaps may be introduced in calculating  
25        sequence identity. The term "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence, wherein the  
30        percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith

and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444, by computerized implementations of these algorithms (GAP, 5 BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

10 "Small molecule" as used herein, is meant to refer to a composition, which has a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries 15 of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts.

A "stem cell" is any cell that, if exposed to proper conditions, is capable of giving rise to two or more different cell types. A "multipotent stem cell" is a stem cell that, if exposed to proper conditions, is capable of giving rise to at least one cell type of two or more different organs or tissues. "Pluripotent stem cells" are 20 pluripotent cells derived from pre-embryonic, embryonic, or fetal tissue at any time after fertilization, and have the characteristic of being capable under the right conditions of producing progeny of several different cell types. Pluripotent stem cells are capable of producing progeny that are derivatives of each of the three germinal layers: endoderm, mesoderm, and ectoderm, according to a 25 standard art-accepted test, such as the ability to form a teratoma in a suitable host. Any cells of primate origin that are capable of producing progeny that are derivatives of all three germinal layers are included in the term "pluripotent stem cell." Included in the definition of pluripotent stem cells are embryonic cells of various types, exemplified by human embryonic stem cells, e.g., as described by 30 Thomson et al. (*Science* 282:1145, 1998); embryonic stem cells from other primates, such as Rhesus or marmoset stem cells, e.g., as described by Thomson et al. (*PNAS*, 92:7844 (1995); *Developmental Biology*, 38:133 (1998)); and human embryonic germ, e.g., as described in Shambloott et al. (*PNAS*, 95:13726 (1998)). A totipotent stem cell is the earliest stem cell in an organism

and is capable of differentiating into any differentiated cell of the organism. A stem cell is said to give rise to another cell if, for example the stem cell differentiates to become the other cell without undergoing cell division, or if the other cell is produced after one or more rounds of cell division and/or  
5 differentiation.

"Substantially pure" refers to an object species that is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50  
10 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the  
15 composition consists essentially of a single macromolecular species.

The term "treating" as used herein is intended to encompass preventing, curing, and/or ameliorating at least one symptom of a condition or disease.

Stem cell cultures are described as "undifferentiated" or "substantially undifferentiated" when a substantial proportion of stem cells and their derivatives  
20 in the population display morphological characteristics of undifferentiated cells, clearly distinguishing them from differentiated cells of embryo or adult origin. Undifferentiated stem cells are easily recognized by those skilled in the art, and typically appear in the two dimensions of a microscopic view with high nuclear/cytoplasmic ratios and prominent nucleoli. It is understood that colonies  
25 of undifferentiated cells within the population will often be surrounded by neighboring cells that are differentiated. Nevertheless, the undifferentiated colonies persist when the population is cultured or passaged under appropriate conditions, and individual undifferentiated cells constitute a substantial proportion of the cell population. Cultures that are substantially undifferentiated  
30 contain at least 20% undifferentiated stem cells, and may contain at least 40%, 60%, or 80%.

A "variant" of a factor, e.g., a growth factor, refers to naturally- or non-naturally-occurring polypeptides that have a certain homology to the factor, e.g., an amino acid sequence homology or a structural homology. An active variant is

a variant that retains at least enough functional activity of the relevant factor that the active variant may be used as a replacement for the factor. Optionally, an active variant retains 25% or more of the activity of the relevant factor.

## 2. Culture methods

5 In certain embodiments, the invention relates to novel methods for culturing stem cells to generate hepatocyte-like cells. The term "stem cells" includes multi-, pluri- and totipotent cells, e.g., embryonic stem cells and adult stem cells, obtained, e.g., from organisms, blastocysts or created by methods such as nuclear transfer and de-differentiation. The stem cells can be  
10 mammalian stem cells, e.g., human, non-human primate, ovine, bovine, porcine, sheep, canine, feline, mink, rabbit, hamster, rat and mouse stem cells.

In one embodiment, the stem cells are embryonic stem (ES) cells. Mouse ES cells were originally obtained from the inner cell mass of pre-implantation embryos, i.e., about 3.5 days old blastocysts (Evans et al. (1981) Nature  
15 292:154-156; Bradley et al. (1984) Nature 309:255-258; Gossler et al. (1986) PNAS 83: 9065-9069; Robertson et al. (1986) Nature 322:445-448). Mouse ES cells from specific strains of mice and methods for obtaining such are described, e.g., in U.S. Pat. Nos. 5,985,659 and 6,190,910 by Kusakabe et al. Human ES cells and methods for obtaining such are described, e.g., in Thomson et al.  
20 (1998) Science 282:114; U.S. Pat. No. 6,200,806 by Thomson et al. and WO 00/27995 by Monash Univ. Non-human primate ES cells and methods for obtaining such are described, e.g., in Thomson et al. (1995) Proc. Natl. Acad. Sci. USA 92:7844 and U.S. Pat. No. 5,843,780 by Thomson et al. ES cells from domestic animals and methods for obtaining such are described, e.g., in WO  
25 90/03432; U.S. Pat. No. 6,107,543 by Sims et al. (bovine ES cells); ES cells from porcines and/or bovines are described, e.g., in Evans et al. (1990) Theriogenology, 33:125 (porcine and bovine ES cells); Notarianni et al. (1990) Proc. 4th World Cong. Genetics Applied to Livestock Production XIII, 58-64 (porcine and ovine ES cells); Notarianni et al. (1991) J. Reprod. Fert. Suppl.  
30 43:255-260 (porcine and sheep ES cells); Piedrahita et al. (1988) Theriogenology, 29:286 (porcine ES cells); Anderson, G. B. (1992) Animal Biotechnology 3(1), 165-175 (livestock ES cells); Stewart, C. L. (1991) Animal Applications of Research in Mammalian Development, Cold Spring Harbor Laboratory Press, New York, pp. 267-283 (domestic animals ES cells); WO

95/16770 (ungulate ES cells) and WO 90/08188 (LIF from livestock). ES cells from other species and methods for obtaining such are described, e.g., in the following publications: Doetschman et al. (1988) *Dev. Biol.*, 127:224 (hamster ES cells) and WO 93 03585 (chicken ES cells).

- 5 Embryonic stem cells of certain species are available publicly or commercially. For example, human ES cells are available from Wisconsin Alumni Research Foundation (WARF, Madison, WI). Mouse ES cells are available from several companies, e.g., Jackson Laboratories (Bar Harbor, ME), the American Type Culture Collection (ATCC, Manassas, VA); and Eurogentech.
- 10 Mouse ES cells are available from various strains of mice. Exemplary mouse ES cells that are commercially available include ES-E14TG2a from mouse strain 129/Ola (CRL-1821; ATCC); ES-D3 [D3] from mouse strain 129/Sv+c/+p (CRL-1934 and CRL-11632; ATCC); ES-D3 GL from mouse strain 129/Sv+c/+p (SCRC-1003; ATCC); ES-C57BL/6 from mouse strain C57BL/6j (SCRC-1002;
- 15 ATCC); 9TR#1 from mouse strain 129/Sv+c/+p having disrupted TNF genes (CRL-11379; ATCC); and TK#1 from mouse strain 129/Sv+c/+p having disrupted IRF-2 genes (CRL-11383; ATCC).

- Human embryonic stem (hES) cells can be prepared as described by Thomson et al. (U.S. Pat. No. 5,843,780; *Science* 282:1145, 1998; *Curr. Top.*
- 20 *Dev. Biol.* 38:133 ff., and 1998; *Proc. Natl. Acad. Sci. USA* 92:7844, 1995). Briefly, human blastocysts are obtained from human in vivo preimplantation embryos. Alternatively, in vitro fertilized (IVF) embryos can be used, or one cell human embryos can be expanded to the blastocyst stage (Bongso et al., *Hum Reprod* 4: 706, 1989). Human embryos are cultured to the blastocyst stage in
- 25 G1.2 and G2.2 medium (Gardner et al., *Fertil. Steril.* 69:84, 1998). Blastocysts that develop are selected for ES cell isolation. The zona pellucida is removed from blastocysts by brief exposure to pronase (Sigma). The inner cell masses are isolated by immunosurgery, in which blastocysts are exposed to a 1:50 dilution of rabbit anti-human spleen cell antiserum for 30 minutes, then washed
- 30 for 5 minutes three times in DMEM, and exposed to a 1:5 dilution of Guinea pig complement (Gibco) for 3 min (see Solter et al., *Proc. Natl. Acad. Sci. USA* 72:5099, 1975). After two further washes in DMEM, lysed trophectoderm cells are removed from the intact inner cell mass (ICM) by gentle pipetting, and the ICM plated on murine endothelial fibroblast (mEF) feeder layers.

After 9 to 15 days, inner cell mass-derived outgrowths are dissociated into clumps either by exposure to calcium and magnesium-free phosphate-buffered saline (PBS) with 1 mM EDTA, by exposure to dispase or trypsin, or by mechanical dissociation with a micropipette; and then replated on mEF in fresh medium. Dissociated cells are replated on mEF feeder layers in fresh ES medium, and observed for colony formation. Colonies demonstrating undifferentiated morphology are individually selected by micropipette, mechanically dissociated into clumps, and replated. ES-like morphology is characterized as compact colonies with apparently high nucleus to cytoplasm ratio and prominent nucleoli. Resulting ES cells are then routinely split every 1-2 weeks by brief trypsinization, exposure to Dulbecco's PBS (without calcium or magnesium and with 2 mM EDTA), exposure to type IV collagenase (about 200 U/mL; Gibco) or by selection of individual colonies by micropipette. Clump sizes of about 50 to 100 cells are optimal.

Human embryonic germ (hEG) cells can be prepared from primordial germ cells present in human fetal material taken about 8-11 weeks after the last menstrual period. Suitable preparation methods are described in Shambloft et al., Proc. Natl. Acad. Sci. USA 95:13726, 1998 and U.S. Pat. No. 6,090,622. Briefly, genital ridges are rinsed with isotonic buffer, then placed into 0.1 mL 0.05% trypsin/0.53 mM sodium EDTA solution (BRL) and cut into  $<1 \text{ mm}^3$  chunks. The tissue is then pipetted through a 100  $\mu\text{L}$  tip to further disaggregate the cells. It is incubated at 37 °C for about 5 min, then about 3.5 mL EG growth medium is added. EG growth medium is DMEM, 4500 mg/L D-glucose, 2200 mg/L mM sodium bicarbonate; 15% ES qualified fetal calf serum (BRL); 2 mM glutamine (BRL); 1 mM sodium pyruvate (BRL); 1000-2000 U/mL human recombinant leukemia inhibitory factor (LIF, Genzyme); 1-2 ng/ml human recombinant basic fibroblast growth factor (bFGF, Genzyme); and 10  $\mu\text{M}$  forskolin (in 10% DMSO). In an alternative approach, EG cells are isolated using hyaluronidase, collagenase, and DNase. Gonadal anlagen or genital ridges with mesenteries are dissected from fetal material, the genital ridges are rinsed in PBS, then placed in 0.1 ml HCD digestion solution (0.01% hyaluronidase type V, 0.002% DNase I, 0.1% collagenase type IV, all from Sigma prepared in EG growth medium). Tissue is minced and incubated 1 h or overnight at 37 °C, resuspended in 1-3 mL of EG growth medium, and plated onto a feeder layer.

Ninety-six well tissue culture plates are prepared with a sub-confluent layer of feeder cells cultured for 3 days in modified EG growth medium free of LIF, bFGF or forskolin, inactivated with 5000 rad  $\gamma$ -irradiation. Suitable feeders are STO cells (ATCC Accession No. CRL 1503). About 0.2 mL of primary germ cell (PGC) suspension is added to each of the wells. The first passage is conducted after 7-10 days in EG growth medium, transferring each well to one well of a 24-well culture dish previously prepared with irradiated STO mouse fibroblasts. The cells are cultured with daily replacement of medium until cell morphology consistent with EG cells are observed, typically after 7-30 days or 1-4 passages.

Mouse ES cells can be obtained, e.g., as described in M.L. Roach and J.D. McNeish (2002) *Methods in Mol. Biol.* 185:1. Briefly, day 3.5 post coitus (p.c.) plugged mice females are sacrificed and the blastocyst stage embryos are flushed from uterine horns. The embryos are washed and transferred onto fresh feeder layers or in media containing about 1,000 units/ml LIF (ESGRO). When the embryos have attached to the dish, the inner cell mass (ICM) is removed from the rest of the embryo and transferred into a dish with fresh media and feeder layers and/or LIF. The next day, the ICM, which should be attached to the dish, is treated with trypsin and split. The cells are then cultured for several days during which the media is changed every day and every second or third day, the colonies are passed. The colonies should not grow larger than 400  $\mu$ m in diameter. The cells are then grown in progressively larger sized dishes.

Other stem cells that can be used include embryonic germ cell lines, e.g., obtained from fetal gonadal tissue or from tissue formed after gestation. Pluripotent human embryonic cell lines derived from cultured human primordial germ cells are described, e.g., in Shambloott et al., *PNAS*, 95:13726 (1998) and PCT International Patent Publication No. WO 98/43679. Primordial germ cells and their isolation are also described, e.g., in U.S. Pat. Nos. 5,453,357 and 5,690,926 by Hogan et al. (mouse and non-mouse primordial germ cells); 6,090,622 by Gearhart and Shambloott (human pluripotential embryonic germ cells) and 6,194,635 by Anderson (porcine primordial germ cells).

In other embodiments, the stem cells are adult stem cells, such as liver stem cells (e.g. oval cells), mesenchymal stem cells, pancreatic stem cells, multipotent adult stem cells and other stem cells that are able to give rise to



hepatocyte-like cells when cultured according to a method described herein. Exemplary stem cells and methods of isolating such are described, e.g., in U.S. Pat. Nos. 5,861,313 by Pang et al. (pancreatic and hepatic progenitor cells); 6,146,889; 6,069,005; and 6,242,252 by Reid et al. (hepatic progenitor cells);  
5 and PCT International Patent Publication Nos. WO 01/11011 (multipotent adult stem cell lines); as well as WO 00/43498 and WO 00/36091 (human liver progenitor cells).

Hepatocyte-like cells of this invention can be genetically altered in a manner that permits the genetic alteration to be either transient, or stable and  
10 inheritable as the cells divide. Undifferentiated cells can be genetically altered and then differentiated into the desired phenotype, or the cells can be differentiated first before genetic alteration. Where the stem cells are genetically altered before differentiation, the genetic alteration can be performed on a permanent feeder cell line that has resistance genes for drugs used to select for  
15 transformed cells, or on stem cells grown in feeder-free culture.

Suitable methods for transferring vector or plasmids into stem cells include lipid/DNA complexes, such as those described in U.S. Pat. Nos. 5,578,475; 5,627,175; 5,705,308; 5,744,335; 5,976,567; 6,020,202; and 6,051,429. Suitable reagents include lipofectamine, a 3:1 (w/w) liposome  
20 formulation of the poly-cationic lipid 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Chemical Abstracts Registry name: N-[2-(2,5-bis[(3-aminopropyl)amino]-1-oxpentyl)amino) ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate), and the neutral lipid dioleoyl  
25 phosphatidylethanolamine (DOPE) in membrane filtered water. Exemplary is the formulation Lipofectamine 2000<sup>TM</sup> (available from Gibco/Life Technologies # 11668019). Other reagents include: FuGENE<sup>TM</sup> 6 Transfection Reagent (a blend of lipids in non-liposomal form and other compounds in 80% ethanol, obtainable from Roche Diagnostics Corp. # 1814443); and LipoTAXI<sup>TM</sup> transfection reagent  
30 (a lipid formulation from Invitrogen Corp., #204110). Transfection of ES cells can also be performed by electroporation, e.g., as described in M.L. Roach and J.D. McNeish (2002) Methods in Mol. Biol. 185:1. Suitable viral vector systems for producing stem cells with stable genetic alterations may be based on

adenoviruses and retroviruses, and may be prepared using commercially available virus components.

In certain embodiments, stem cells can be stably transfected with a marker that is under the control of a hepatocyte-specific regulatory region, such that during differentiation, the marker is selectively expressed in the hepatocyte-like cells, thereby allowing selection of the hepatocyte-like cells relative to the cells that do not express the marker. The marker can be, e.g., a cell surface protein or other detectable marker, or a marker that can make cells resistant to conditions in which they die in the absence of the marker, such as an antibiotic resistance gene. These methods are further described, e.g., in U.S. Patent No. 6,015,671. Hepatocyte specific promoters include the promoter of late stage hepatocyte markers, e.g., as described herein. Accordingly, hepatocyte-like cells can be further purified by selection of the cells expressing such a marker, e.g., by selection on a medium that kills cells that do not express the marker (e.g., in the presence of an antibiotic if the marker is an antibiotic resistance marker), or by selecting cells that are positive for the marker by, e.g., fluorescence activated cell sorting (FACS), panning or using beads.

In certain embodiments, stem cells are exposed to one or more culture conditions in an appropriate sequence and for an appropriate time so as to generate a cell population comprising hepatocyte-like cells. In certain embodiments, one or more of the culture conditions comprises one or more of the following growth factors: aFGF, EGF, HGF, OSM, HPO, nicotinamide, dexamethasone, insulin and transferrin. Optionally, the method comprises exposing the cells to an early culture condition comprising aFGF and EGF; a middle culture condition comprising HGF; and/or a late culture condition comprising oncostatin M. The factors used in the early, middle and late conditions need not be mutually exclusive, and cells need not be exposed to these conditions in an uninterrupted succession. Cells may be frozen or otherwise stored between various steps, and cells may be exposed to intervening culture conditions, so long as the intervening culture conditions do not disrupt the program of differentiation caused by the combination of early, middle and late conditions.

In certain embodiments, the cells are exposed to an early culture condition comprising EGF and/or aFGF; a middle culture condition comprising

EGF, aFGF and/or HGF; and a late culture condition comprising EGF, HGF and/or OSM.

In another embodiment, the cells are exposed to an early culture condition comprising EGF, aFGF and/or nicotinamide; a middle culture condition  
5 comprising EGF, aFGF, HGF and/or nicotinamide; and a late culture condition comprising EGF, HGF, OSM, nicotinamide, insulin, transferrin, selenium-G and/or dexamethasone.

In certain embodiments, cells are exposed to culture conditions comprising HPO, e.g., HPO is added to a middle and/or late culture condition.

10 The precise concentration of growth factor to be used in any one culture condition may be optimized and may vary depending on the source of growth factor and the form (e.g. purified from a natural source, produced as a recombinant form, a fragment or variant or a functional mimic). In an exemplary embodiment, EGF may be used at a concentration ranging from about 1-50  
15 ng/ml; preferably from about 1-20 ng/ml; even more preferably from about 5-15 ng/ml; and most preferably about 10 ng/ml. In an exemplary embodiment, aFGF may be used at a concentration ranging from about 1-50 ng/ml; preferably about 1-20 ng/ml; even more preferably about 5-15 ng/ml; and most preferably about 10 ng/ml. In an exemplary embodiment, HGF may be used at a concentration  
20 ranging from about 5-100 ng/ml; more preferably about 5-50 ng/ml; even more preferably about 15-30 ng/ml; and most preferably about 25 ng/ml. In an exemplary embodiment, OSM may be used at a concentration of 1-50 ng/ml; more preferably about 1-30 ng/ml; even more preferably about 5-15 ng/ml; and most preferably about 10 ng/ml. In an exemplary embodiment, HPO may be  
25 used at a concentration ranging from about 10-250 ng/ml; more preferably about 20-100 ng/ml; even more preferably about 40 to 60 ng/ml; and most preferably about 50 ng/ml. In an exemplary embodiment, nicotinamide may be used at a concentration ranging from about 1-50  $\mu$ M more preferably about 1-30  $\mu$ M; even more preferably about 5-15  $\mu$ M; and most preferably about 10  $\mu$ M. In an  
30 exemplary embodiment, dexamethasone may be used at a concentration of 20-500 nM; preferably about 20-200 nM; even more preferably about 80-120 nM; and most preferably about 100 nM. In an exemplary embodiment, insulin may be used at a concentration of about 0.1-100  $\mu$ g/ml; preferably about 1-50  $\mu$ g/ml; even more preferably about 5-20  $\mu$ g/ml; and most preferably about 10  $\mu$ g/ml. In

an exemplary embodiment, transferrin may be used at a concentration of 0.1-100  $\mu$ g/ml; preferably about 1-50  $\mu$ g/ml; even more preferably about 1-10  $\mu$ g/ml; and most preferably about 5  $\mu$ g/ml. Transferrin in HepEB medium is preferably present at a concentration of about 10 to about 1000  $\mu$ g/ml; more  
5 preferably about 100 to about 1000  $\mu$ g/ml; even more preferably about 200 to about 500  $\mu$ g/ml; and most preferably about 300  $\mu$ g/ml. In an exemplary embodiment, selenium may be used at a concentration of 0.1-100 ng/ml; preferably about 1-50 ng/ml; even more preferably about 1-10 ng/ml; and most preferably about 5 ng/ml.

10        Optionally, polypeptide growth factors are matched to the species of the cells. For example, it may be desirable to use human EGF when working with human cells and murine EGF when working with murine cells.

In addition to the appropriate growth factors, other media components may be selected as appropriate for the cellular starting material, and some  
15 degree of routine optimization is expected for each culture situation. For example, commonly used media bases include Dulbecco's Modified Eagle's Medium (DMEM), Ham's F-12 nutrient mixture, Iscove's Modified Dulbecco's Medium (IMDM), McCoy's 5A, RPMI 1640, etc. Generally, differences between the different media can be compensated for with the addition or omission of  
20 supplements, such as carbon sources (e.g. glucose, pyruvate, etc.), serum (e.g. fetal bovine serum), vitamins, amino acids, etc. Other media components that may be selected and optimized to match the desired culture conditions are antibiotics (e.g. aminoglycosides such as gentamycin, penicillins, etc.) amino acids (particularly glutamine) and reducing agents (e.g. thiols such as  
25 monothioglycerol).

In certain embodiments, the cells are cultured with one or more of the Hep EB, Hep I, Hep II, Hep III, Hep IV media described in the Examples below or variants thereof.

Each of the differentiation steps described herein can be conducted for a  
30 time appropriate to get the cells ready for the next differentiation step. Generally, each differentiation step takes from 2-4 days, preferably 3 days.

In a particular embodiment, differentiation of ES cells is conducted as follows. ES cells cultured in the presence of a feeder layer and/or leukemia inhibitory factor (LIF) are removed from the feeder layer and/or LIF, such as to

allow differentiation. During this first stage, referred to as the "embryoid body stage," ES cells form embryoid bodies. This first stage extends from day 0 to about day 5, with day 0 corresponding to the day the feeder layer and/or LIF is removed, such that differentiation may begin. The cells may be cultured in media, e.g., containing transferrin, e.g., the HepEB media described in the Examples. In a second stage, referred to as the "early stage," consisting of about day 6 to day 8, the embryoid bodies are cultured in a medium comprising EGF, aFGF, and optionally nicotinamide. For example, the cells can be cultured in the medium HepI described in the Examples. Towards the end of this stage, e.g., day 8, the embryoid bodies may be very spread and may be touching each other. At this point, the embryoid bodies are dissociated into single cells and cultured in the same medium as prior to the dissociation. In a third stage, referred to as the "middle stage," consisting of about day 9 to day 11, the cells are cultured in a medium comprising HGF, and optionally EGF, aFGF, and/or nicotinamide. For example, the cells can be cultured in the medium HepII described in the Examples. During this stage, the cells form a nice monolayer that is about 60-70% confluent. Cells may be passed during this stage, e.g., on day 11. In a fourth stage, referred to as the "late stage," consisting of about day 12-14, the cells are cultured in a medium comprising OSM, and optionally EGF, HGF, dexamethasone, insulin, transferrin, and/or selenium-G. For example, the cells can be cultured in the medium HepIII described in the Examples. During this stage, the cells appear flatter, more epithelial-like in morphology and 60-70% confluent. The cells may be passed during this stage, e.g., on day 14.

During the various culture steps of the ES cells and derivatives thereof, when the cells are not cultured in the presence of feeder layers and/or LIF, the cells may be cultured on dishes coated with collagen, e.g., collagen type I. For example, cell can be cultured on coated dishes from the moment they start forming embryoid bodies. In one embodiment, the cells are cultured on non-coated dishes until about day 5, at which point the cells which are in the form of embryoid bodies are transferred to collagen type I coated dishes. Other coatings that may be used include fibronectin, e.g., 0.1  $\mu$ g/ml, and Matrigel (containing a mixture of extracellular matrix (ECM) components), e.g., 1% Matrigel.

Differentiation of ES cells can also be promoted by withdrawing serum or serum replacement from medium, withdrawing a factor that promotes

proliferation, withdrawing a factor that inhibits differentiation, or adding a new factor that promotes differentiation.

When cells which are not ES cells are used for differentiation into hepatocyte-like cells, the first stage of differentiation may correspond to the  
5 second or third stage of differentiation of ES cells, i.e., the early or middle stage, respectively. The cells may then be taken through the later steps of differentiation described above for the ES cells.

The differentiation of cells can be monitored by visual inspection of the cells. The differentiation can also be monitored by analysis of phenotypic or  
10 functional characteristics of ES cells, hepatocytes and precursors thereof. For example, differentiation can be monitored by analysis of expression of early and late markers of hepatocyte differentiation. Exemplary early markers include hepatocyte nuclear factor (HNF)-3 $\beta$ , GATA4, CK19 and  $\alpha$ -fetoprotein, as described, e.g., in Schwartz et al. (2002) J. Clin. Invest. 109: 1291. Late  
15 markers of hepatocyte differentiation include CK18, albumin and HNF-1 $\alpha$  (see, e.g., Schwartz et al., *supra*). Other tests that can be used are further described herein, in particular, in the Examples.

In another embodiment, the invention relates to methods for selecting hepatocyte-like cells from a population of cells, e.g., a population of cells  
20 obtained from the differentiation of stem cells as described above. In one embodiment, a cell population comprising, or suspected of comprising, a hepatocyte-like cell is placed in a culture condition that favors the growth or survival of hepatocytes, e.g., by selecting for gluconeogenic cells over non-gluconeogenic cells. It is generally accepted that only two types of cells in the  
25 human body are capable of performing gluconeogenesis: hepatocytes and mammary gland epithelial cells. Accordingly, a culture condition that favors the growth or survival of gluconeogenic cells will tend to enrich (or select) for hepatocytes versus essentially all other cell types. Conditions that favor growth or survival of gluconeogenic cells include, for example, conditions where the  
30 most significant, or, optionally, the sole carbon source is a carbon source that supports the growth of gluconeogenic cells but not non-gluconeogenic cells. An exemplary carbon source of this type is pyruvate. Accordingly, a medium containing reduced amounts of glucose, or no glucose at all, and pyruvate will tend to favor the growth or survival of gluconeogenic cells. Compounds that can

be converted to pyruvate may also be used. An exemplary medium for selecting hepatocyte-like cells comprises sufficient pyruvate to permit survival or growth of gluconeogenic cells and contains insufficient nutrients (e.g. glucose) to support the survival or growth of non-gluconeogenic cells. Exemplary pyruvate concentrations range from about 0.1-30 mM; more preferably about 0.1-10 mM; even more preferably about 0.2-5mM; and most preferably about 1 mM. In certain embodiments pyruvate is supplied as pyruvic acid.

In an exemplary embodiment, a stem cell is differentiated into a hepatocyte-like cell as described above, e.g., by culture through stages 1-4 described above, and then subjected to the enrichment step described above, which is also referred to as a "maturation and selection stage" (stage 5). For example, ES cells subjected to stages 1-4 of differentiation can then be subjected to the maturation and selection stage (stage 5), consisting of about day 15-18. During this stage, the cells are cultured in a medium that is selective for gluconeogenic cells, as described herein and known to those of skill in the art. For example, the cells can be cultured in a medium comprising pyruvic acid or pyruvate and optionally one or more of EGF, HGF, OSM, dexamethasone, and/or sodium butyric acid. In a preferred embodiment, the cells can be cultured in the medium HepIV described in the Examples. Towards the end of this stage, a lot of cell death will be observed. The medium can be removed and the cells washed every other day or every day. On day 19 or 20, the cells can be washed and further incubated in a glucose containing medium, comprising, e.g., EGF, HGF, and/or OSM, such as the medium HepIII described in the Examples.

In a further embodiment, butyrate is employed as an agent that favors the retention of hepatocyte-like cells, as evidenced by the retention of hepatocyte characteristics. Exemplary media comprise butyrate at concentrations ranging from 0.1 mM to 25 mM; preferably about 1-15 mM; more preferably about 2-10mM; and most preferably about 5 mM. In certain embodiments butyrate is provided as sodium butyrate. Dimethylsulfoxide (DMSO) may be used in a similar manner. For example, a medium may comprise 2-50 mg/ml DMSO; preferably about 5-30mg/ml; more preferably about 5-20mg/ml and most preferably about 10 mg/ml.

In an additional embodiment, a cell population comprising, or suspected of comprising, hepatocyte-like cells is exposed to a gluconeogenic medium

comprising butyrate or DMSO. Optionally, the medium comprises pyruvate and butyrate or DMSO at the ranges of concentrations described above. HEP IV, described below in the examples, is an exemplary medium of this type.

In yet a further embodiment, methods of the invention may include  
5 exposing cells to a culture condition that is suitable for activation of hepatocyte-like cells, or, in other words, increasing the level of one or more hepatocyte metabolic activities. For example, phenobarbital and chemically related compounds are known to induce the expression of one or more cytochrome P450 enzymes in hepatocytes, particularly in human hepatocytes. Pyrethroids  
10 (e.g. permethrin, cypermethrin, and fenvalerate) may be used in a similar manner. Heder et al. *Biochem Pharmacol* 2001 62(1):71-9. Pregnenolones, such as pregnenolone 16 $\alpha$ -carbonitrile also induce the expression or activity of various cytochrome P450 enzymes, particularly in rodent cells. Dexamethasone may be used similarly. In certain embodiments, pregnenolone 16 $\alpha$ -carbonitrile  
15 is used at a concentration ranging from 10 nM to 1 mM; preferably about 50-500 nM; more preferably about 90-200 nM; and most preferably about 100 nM. For example, cells can be subjected to this stage (stage 6) by incubation in a medium, e.g., HepIII, comprising one such agent. After one day of incubation, the medium of the cells can be replaced by a medium comprising one or more of  
20 OSM, nicotinamide, dexamethasone, insulin, transferrin, and selenium. An exemplary medium is medium HepV described in the Examples.

In general, cells are exposed to a condition that activates hepatocyte-like cells at a stage when an enriched population of hepatocyte-like cells has been obtained.

25 Certain methods described herein employ polypeptide growth factors. Preparations of each of these factors are commercially available (with the exception of HPO), and sources from which they can be obtained are provided in the Examples. It is also understood that one may produce these factors according to methods known in the art. Exemplary nucleotide and amino acid  
30 sequences for these factors are provided in the attached sequence listing and are further described herein. For simplicity, the nucleotide and amino acid sequences of the various growth factors, described herein, and corresponding GenBank Accession Numbers, if any, have the following SEQ ID NOs:



**Table 1**  
**SEQ ID NOs of factors described herein**

	Sequence Accession	SEQ ID NO	GenBank Number (if any)
5	Human aFGF nucleotide sequence	SEQ ID NO: 1	NM_000800
	Human aFGF amino acid sequence	SEQ ID NO: 2	NP_000791.1
	Mouse aFGF nucleotide sequence	SEQ ID NO: 3	M30641
	Mouse aFGF amino acid sequence	SEQ ID NO: 4	AAA37618.1
10	Human EGF nucleotide sequence	SEQ ID NO: 5	NM_001963
	Human EGF amino acid sequence	SEQ ID NO: 6	NP_001954.1
	Mouse EGF nucleotide sequence	SEQ ID NO: 7	J00380
	Mouse EGF amino acid sequence	SEQ ID NO: 8	AAA37539.1
	Human HGF nucleotide sequence	SEQ ID NO: 9	M29145
15	Human HGF amino acid sequence	SEQ ID NO: 10	AAA52650.1
	Mouse HGF nucleotide sequence	SEQ ID NO: 11	D10212
	Mouse HGF amino acid sequence	SEQ ID NO: 12	BAA01064.1
	Human partial HPO nucleotide sequence	SEQ ID NO: 13	AF306863
	Human partial HPO amino acid sequence	SEQ ID NO: 14	AAG38105
20	Mouse partial HPO nucleotide sequence	SEQ ID NO: 15	AF148688
	Mouse partial HPO amino acid sequence	SEQ ID NO: 16	AAD36987
	Mouse full length HPO nucleotide sequence	SEQ ID NO: 17	see
	Sequence Listing		
	Mouse full length HPO amino acid sequence	SEQ ID NO: 18	see
25	Sequence Listing		
	Human OSM nucleotide sequence	SEQ ID NO: 19	BC011589
	Human OSM amino acid sequence	SEQ ID NO: 20	AAH11589.1
	Mouse OSM nucleotide sequence	SEQ ID NO: 21	J04806
	Mouse OSM amino acid sequence	SEQ ID NO: 22	AAA57265.1

30

Regarding HPO, the short form or the full length form may be used in differentiation. A nucleic acid encoding a human homolog of the full length form of HPO can be isolated, e.g., by PCR using primers based on the sequence set forth in AF306863, AF183892 and SEQ ID NO: 17.

The factors are encoded as a precursor protein, a portion of which becomes the mature factor. The location of the signal peptide for each of these is known in the art. A person of skill in the art will readily recognize that variants, fragments, functional mimics and orthologs can be used, provided that such  
5 compounds can be provided at a concentration sufficient to provide similar functional activity.

For example, a variant may be generated by making conservative amino acid changes and testing the resulting variant in one of the functional assays described above or another functional assay known in the art. Conservative  
10 amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of  
15 amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine,  
20 alanine-valine, and asparagine-glutamine.

As those skilled in the art will appreciate, variants or fragments of polypeptide growth factors can be generated using conventional techniques, such as mutagenesis, including creating discrete point mutation(s), or by truncation. For instance, mutation can give rise to variants which retain  
25 substantially the same, or merely a subset, of the biological activity of a polypeptide growth factor from which it was derived.

Growth factor variants may also be chemically modified by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives can  
30 be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

Functional mimics of a growth factor include any compound that has an effect on at least a portion of the cellular signaling pathway of the relevant

growth factor and is able to elicit a similar response in a functional assay for the growth factor, such as in one of the assays disclosed herein. As with fragments and variants, a functional mimic need not have the same concentration range for effectiveness, so long as the functional mimic is sufficiently active and non-toxic that there exists a practical concentration at which it can be used. A functional mimic may be generated by, for example, designing a molecule that activates the growth factor receptor, i.e., an EGF functional mimic could be a molecule that activates the EGF receptor.

For further elaboration of general techniques useful in the practice of this invention, the practitioner can refer to standard textbooks and reviews in cell biology, tissue culture, and embryology. Included are Teratocarcinomas and embryonic stem cells: A practical approach (E. J. Robertson, ed., IRL Press Ltd. 1987); Guide to Techniques in Mouse Development (P. M. Wasserman et al., eds., Academic Press 1993); Embryonic Stem Cell Differentiation in Vitro (M. V. Wiles, Meth. Enzymol. 225:900, 1993); Properties and uses of Embryonic Stem Cells: Prospects for Application to Human Biology and Gene Therapy (P. D. Rathjen et al., al., 1993). Differentiation of stem cells is reviewed in Robertson, Meth. Cell Biol., 75:173 (1997); as well as Pedersen, Reprod. Fertil. Dev., 10:31 (1998).

Proteins can be produced, e.g., by expression of a nucleic acid encoding the protein in a eukaryotic or prokaryotic system or in an in vitro translation system according to techniques well known in the art. It is preferable to express a protein in a eukaryotic system, such that the protein has the proper posttranslational modifications.

Human HPO can be produced recombinantly as described, e.g., in Yang et al., Acta Biochim. Biophys. Sin., 29:414 (1997).

EGF activity may be tested by measuring the ability of a compound to stimulate <sup>3</sup>H-thymidine incorporation in an EGF-responsive mouse fibroblast cell line, such as the Balb/3T3 cell line. Rubin et al., PNAS, 88:415 (1991). In this type of assay, human recombinant EGF will have an ED<sub>50</sub> typically in the range of 0.1–0.4 ng/ml. An EGF variant, fragment or functional mimic need not have a similar ED<sub>50</sub>, but the activity should be sufficiently high that the compound can be used in a culture medium at a reasonable concentration. A functional mimic

for EGF may be, for example, a compound that is an agonist for an EGF receptor.

HGF activity may be tested by measuring the ability of a compound to stimulate  $^3\text{H}$ -thymidine incorporation in the HGF-responsive monkey epithelial cell line, 4MBr-5. Rubin et al., PNAS, 88:415 (1991). In this type of assay, human recombinant HGF will have an  $\text{ED}_{50}$  typically in the range of 20 – 40 ng/ml. An HGF variant, fragment or functional mimic need not have a similar  $\text{ED}_{50}$ , but the activity should be sufficiently high that the compound can be used in a culture medium at a reasonable concentration. A functional mimic for HGF may be, for example, a compound that is an agonist for an HGF receptor.

OSM activity may be tested by measuring the ability of a compound to stimulate  $^3\text{H}$ -thymidine incorporation in quiescent NIH/3T3 cells. In this type of assay, mouse recombinant OSM will have an  $\text{ED}_{50}$  typically in the range of 2 – 4 ng/ml. Human OSM activity may also be tested by measuring proliferation of a factor-dependent human erythroleukemic cell line, TF-1. Kitamura et al., J. Cell Physiol., 140:323-34 (1989). In this type of assay, human recombinant OSM will have an  $\text{ED}_{50}$  typically in the range of 0.1 - 3 ng/ml. An OSM variant, fragment or functional mimic need not have a similar  $\text{ED}_{50}$ , but the activity should be sufficiently high that the compound can be used in a culture medium at a reasonable concentration. A functional mimic for OSM may be, for example, a compound that is an agonist for an OSM receptor.

aFGF activity may be tested by measuring the ability of a compound to stimulate  $^3\text{H}$ -thymidine incorporation in an aFGF-responsive mouse fibroblast cell line, such as the Balb/3T3 cell line. Rubin et al., PNAS, 88:415 (1991). In this type of assay, human recombinant aFGF will have an  $\text{ED}_{50}$  typically in the range of 2 - 10 ng/ml. An aFGF variant, fragment or functional mimic need not have a similar  $\text{ED}_{50}$ , but the activity should be sufficiently high that the compound can be used in a culture medium at a reasonable concentration. A functional mimic for aFGF may be, for example, a compound that is an agonist for an aFGF receptor.

HPO activity may be tested by measuring the ability of a compound to stimulate  $^3\text{H}$ -thymidine incorporation in an HPO-responsive hepatocyte cell line, such as the HepG2 cell line, as described, e.g., in Wang et al., J. Biol. Chem., 274:11469 (1999). An HPO variant, fragment or functional mimic should have

an activity sufficiently high that the compound can be used in a culture medium at a reasonable concentration. A functional mimic for HPO may be, for example, a compound that is an agonist for an HPO receptor, which is described, e.g., in Wang et al., supra.

5     3.     Hepatocyte-like cells

The invention provides enriched populations of hepatocyte-like cells. Exemplary populations of cells comprise at least about 50%; preferably at least about 60%; 70%; 80%; 90%; 95%; 98% and most preferably 99% of hepatocyte-like cells. As set forth in the Examples, the methods described herein, e.g.,  
10    differentiation through stages 1-4, permit the obtention of a population of cells in which at least about 50% of the cells are hepatocyte-like cells. When a maturation and selection step was added, populations of at least about 90% of hepatocyte-like cells were obtained.

Hepatocyte-like cells can be characterized as follows. The cells may also  
15    be positive for late stage markers of hepatocytes, such as HNF-1 $\alpha$ , cytokeratin (CK)18 and albumin; the absence of early hepatocyte markers, e.g., HNF-3 $\beta$ , GATA4, CK19,  $\alpha$ -fetoprotein; express cytochrome P450 genes, e.g., CYP1A1, CYP2B1, CYP2C6, CYP2C11, CYP2C13, CYP3A2 and CYP4A1; and acquire a polarized structure. Hepatocyte-like progenitor cells may be detected by the  
20    presence of early hepatocyte markers. Other markers of interest for liver cells include  $\alpha$ 1-antitrypsin, glucose-6-phosphatase, transferrin, asialoglycoprotein receptor (ASGR), CK7,  $\gamma$ -glutamyl transferase; HNF 1 $\beta$ , HNF 3 $\alpha$ , HNF-4 $\alpha$ , transthyretin, CFTR, apoE, glucokinase, insulin growth factors (IGF) 1 and 2, IGF-1 receptor, insulin receptor, leptin, apoAII, apoB, apoCIII, apoCII, aldolase  
25    B, phenylalanine hydroxylase, L-type fatty acid binding protein, transferrin, retinol binding protein, and erythropoietin (EPO).

Tissue-specific markers can be detected by immunological techniques, such as flow immunocytochemistry for cell-surface markers, immunohistochemistry (for example, of fixed cells or tissue sections) for  
30    intracellular or cell-surface markers, Western blot analysis of cellular extracts, and enzyme-linked immunoassay, for cellular extracts or products secreted into the medium. The expression of tissue-specific gene products can also be detected at the mRNA level by Northern blot analysis, dot-blot hybridization analysis, or by reverse transcriptase initiated polymerase chain reaction (RT-

PCR) using sequence-specific primers in standard amplification methods. Sequence data for the particular markers listed in this disclosure can be obtained from public databases such as GenBank (URL [www.ncbi.nlm.nih.gov:80/entrez](http://www.ncbi.nlm.nih.gov:80/entrez)). Primers for amplifying sequences of marker of interest can also be found, e.g., in

5 Schwartz et al. (2002) J. Clin. Invest. 109:1291. Expression of tissue-specific markers as detected at the protein or mRNA level is considered positive if the level is at least 2-fold, and preferably more than 10- or 50-fold above that of a control cell, such as an undifferentiated stem cell, a fibroblast, or other unrelated cell type.

10 Hepatocyte-like cells may also display the following biological activities, as evidenced by functional assays. The cells may have a positive response to dibenzylfluorescein (DBF) (see Examples); have the ability to metabolize certain drugs, e.g., dextromethorphan and coumarin; have drug efflux pump activities (e.g., P glycoprotein activity); upregulation of CYP activity by phenobarbital, as

15 measured, e.g., with the pentoxyresorufin (PROD) assay, which is seen only in hepatocytes and not in other cells (see, e.g., Schwartz et al., J. Clin. Invest., 109:1291 (2002)); take up LDL, e.g., Dil-acil-LDL (see, e.g., Schwartz et al., supra); store glycogen, as determined, e.g., by using a periodic acid-Schiff (PAS) staining of the cells (see, e.g., Schwartz et al., supra); produce urea and albumin

20 (see, e.g., Schwartz et al., supra); and present evidence of glucose-6-phosphatase activity.

Hepatocyte-like cells may be characterized for drug efflux pump activity (e.g., P glycoprotein activity) by measuring the accumulation of various test compounds in cells that have been treated or not treated with an inhibitor of P-

25 glycoprotein. Cells that have P-glycoprotein activity are expected to show greater cellular accumulation of the test compound in the absence of the P-glycoprotein inhibitor than in the presence of the inhibitor.

Diazepam and 7-EC metabolic activity can be measured as follows.  $4 \times 10^6$  hepatocyte-like cells are cultured in a monolayer in 5 ml of medium

30 containing 50  $\mu\text{g/ml}$  diazepam or 7-EC and the amount of diazepam or 7-hydroxycoumarin metabolites present in the culture supernatant measured after 3 hours of culture, respectively. Diazepam and 7-hydroxycoumarin metabolites can be assayed by high performance liquid chromatography (HPLC) using a C18

$\mu$ -Bondpack reverse phase column according to known methods, e.g., Jauregui et al., *Xenobiotica*, 21:1091-106 (1991).

Acetaminophen and its metabolites can be determined by ion-pairing HPLC using a C18 reverse phase column. Acetaminophen metabolism can be measured as follows.  $4 \times 10^6$  hepatocyte-like cells are cultured in a monolayer in 5 ml of medium containing 5 mM acetaminophen (0.756 mg/ml), and the amount of acetaminophen glucuronide present in the culture supernatant measured after 3 hours of culture. The amount of acetaminophen and its metabolites, e.g., acetaminophen glucuronide, can be determined by ion-pairing high performance liquid chromatography, e.g, using the method of Colin et al., *J. Chromatogr.*, 377:243-51 (1986). Acetaminophen may also be metabolized via a sulfonation pathway and metabolites may be assayed using methods known in the art.

Lidocaine metabolism can be measured using known methods, e.g., Jauregui et al., *Hepatology*, 21:460-69 (1995). For example,  $4 \times 10^6$  hepatocyte-like cells are cultured in a monolayer in 5 ml of medium containing 20  $\mu$ g/ml lidocaine, and the amount of lidocaine metabolite, e.g., monoethylglycinexylidide (MEGX), present in the culture supernatant is measured after 3 hours of culture. Metabolism of lidocaine can be tested using a TDX Analyzer manufactured by Abbott Diagnostics Laboratories, No. Chicago, Ill.

Ammonia metabolism can be measured according to methods known in the art, e.g., using the commercial analyzer, Ektachem, manufactured by Kodak Corp. Rochester, N.Y. Ammonia metabolism can be detected by measuring the amount of ammonia remaining in the culture supernatant after 3 hours of culture.

#### 4. Uses for purified hepatocyte preparations

In one embodiment, hepatocyte-like cells are used for testing whether test compounds (or agents) have a biological effect, e.g., a cytotoxic effect, on hepatocytes. For example, a hepatocyte-like cell preparation is incubated in the presence or absence of a test compound for a time sufficient to determine whether the compound may have a biological effect on the cells, preferably under physiological conditions, and determining whether the test compound had a biological effect on the cells, relative to the cells that were not treated with the test compound. Cells can be incubated with various concentrations of a test compound. In an illustrative embodiment, cells are plated in the wells of a multi-well plate to which different concentrations of the test compound are added, e.g.,

0  $\mu$ M; 0.01  $\mu$ M; 0.1  $\mu$ M; 1  $\mu$ M; 10  $\mu$ M; 100  $\mu$ M; 1 mM; 10 mM and 100 mM. Cells can be incubated for various times, e.g., 1 minute, 10 minutes, 1 hour, 2 hours, 5 hours, 10 hours, 24 hours, 36 hours or more.

5 The biological effect that is measured can be triggering of cell death (i.e., cytotoxicity or hepatotoxicity); a cytostatic effect; or a transforming effect on the cell, as determined, e.g., by an effect on the genotype or phenotype of the cells. The cytotoxicity on cells can be determined, e.g., by incubating the cells with a vital stain, such as trypan blue.

10 Such screening assays can easily be adapted to high throughput screening assays.

Hepatocyte-like cells can also be used for metabolic profiling. In one embodiment, cells or a fraction thereof, e.g., a microsome fraction, are contacted with a test agent, potentially at different concentrations and for different times, the media is collected and analyzed to detect metabolized forms of the test agent. Optionally, a control molecule, such as bufuralol is also used. Metabolic  
15 profiling can be used, e.g., to determine whether a subject metabolizes a particular drug and if so, how the drug is metabolized. For such assays, it is preferable that the hepatocyte-like cells used derive from the subject.

20 The hepatocyte-like cells of this invention may also be used to screen candidate compounds or environmental conditions that, e.g., affect differentiation or metabolism of the cells. The hepatocyte-like cells may further be used to obtain cell specific antibody preparations and cell-specific cDNA libraries, e.g., to study patterns of gene expression, or as an active ingredient in a pharmaceutical preparation.

25 In another embodiment, hepatocyte-like cells are administered to a subject in need thereof. The cells can be administered to the liver of the subject, e.g., for tissue reconstitution or regeneration. The cells may be administered in a manner that permits them to graft to the intended tissue site and reconstitute or regenerate the functionally deficient area. Prior to administration, the cells may  
30 be modified to suppress an immune reaction from the subject to the cells or vice-versa (graft versus host disease), according to methods known in the art.

Hepatocyte-like cells may be administered to a subject having a complete or partial liver failure, such as resulting from a hepatitis C infection.



Hepatocytes-like cells can be assessed in animal models for ability to repair liver damage. One such example is damage caused by intraperitoneal injection of D-galactosamine (Dabeva et al., Am. J. Pathol., 143:1606 (1993)). Efficacy of treatment can be determined by immunocytochemical staining for  
5 liver cell markers, microscopic determination of whether canalicular structures form in growing tissue, and the ability of the treatment to restore synthesis of liver-specific proteins.

Cell compositions for administration to a subject in accordance with the present invention thus may be formulated in any conventional manner using one  
10 or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. Hepatocyte-like cells can be used in therapy by direct administration, or as part of a bioassist device that provides temporary liver  
15 function while the subject's liver tissue regenerates itself following fulminant hepatic failure. For general principles in medicinal formulation, the reader is referred to Cell Therapy: Stem Cell Transplantation, Gene Therapy, and Cellular Immunotherapy, by G. Morstyn & W. Sheridan eds, Cambridge University Press, 1996; and Hematopoietic Stem Cell Therapy, E. D. Ball, J. Lister & P. Law,  
20 Churchill Livingstone, 2000. The compositions may be packaged with written instructions for use of the cells in tissue regeneration, or restoring a therapeutically important metabolic function.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology,  
25 transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide  
30 Synthesis (M. J. Gait ed., 1984); Mullis et al., U.S. Patent No. 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the

treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

10

### EXAMPLES

The present invention is further illustrated by the following examples which should not be construed as limiting in any way.

#### Example 1: Maintenance of Embryonic Stem Cells

This example describes methods used for thawing, feeding, subculturing, and freezing ES cells, as well as removing ES cells from feeder cell layers.

ES cells were thawed as follows. First a 100mm plate with feeder cells ("feeder plate") was prepared as follows. The feeder cells used were primary embryonic fibroblasts (PEF) cells prepared as described in "Manipulating the mouse embryo" by Brigid Hogan, Fank Costantini and Elizabeth Lacy, Cold Spring Harbor Laboratory 1986. Several days prior to thawing ES cells, feeder cells were plated onto 100 mm dishes as described in E.J. Robertson "Embryo-derived stem cell lines, in Teratocarcinomas and embryonic stem cells: a practical approach, E.J. Robertson, editor, IRL Press, Washington D.C., 1987. The day of thawing of ES cells, the media of the feeder plates was removed, the feeder cells were washed with 10ml PBS, and 15 ml of the following media was added to each plate: stem cell media SCML consisting of KO-DMEM (Gibco/Invitrogen) to which the following ingredients were added: 15% FBS (Gibco/Invitrogen); 0.2mM L-Glutamine (Gibco/Invitrogen); 0.1mM MEM nonessential amino acids (Gibco/Invitrogen); 0.1mM 2-Mercaptoethanol (Sigma); 1000units/ml ESGRO (also known as Leukemia Inhibitory Factor or LIF) (Chemicon); and either 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin or 20ng/ml gentamycin (all from Gibco/Invitrogen).

Prior to using a feeder plate, it was determined whether the feeder cells were healthy. Primary embryonic fibroblast feeders usually last about 7-10 days. The prepared feeder was placed back into the incubator to equilibrate.

5 A vial of ES cells containing enough cells to plate one 100mm dish with an even spread of colonies (approximately  $2-3 \times 10^6$  cells) was removed from -150°C, plunged into a 37°C water-bath, and the vial was agitated until the frozen suspension became a slurry. The vial was doused with alcohol and transferred to a tissue culture hood. The cell suspension was transferred from the vial to the prepared feeder plate. The plate was swirled to evenly distribute the ES cells  
10 over the entire feeder surface, and returned to the incubator.

The next morning, the media was removed and replaced with fresh SCML. The dish was returned to the incubator and cultured another day. If the cells recovered easily from the freeze/thaw, they were generally ready to be split 48 hours after thawing.

15 ES cell cultures were daily fed as follows. The dishes were examined for the condition of the ES cell colonies and observations were recorded. Colony morphology was monitored as a gauge of culture conditions. Healthy ES cell colonies tended to have smooth borders, and the cells were tightly packed together so the individual cells were not detectable, and the entire colony has  
20 depth so as to give a refractile ring around it. Media was removed from the healthy cells and replaced with SCML.

ES cells were subcultured as follows. The plates were fed approximately 1-2 hours prior to passage. Media was removed and replaced with fresh SCML and the dish was returned to the incubator. The cells in the dish were examined  
25 for colony morphology, density and size. As a guideline, an even distribution of colonies over the entire dish, averaging 200-400µm in diameter and spaced 200-400µm apart, was split 1:8. The ratio for splitting the cells was calculated, generally so as to plate  $1.5-2 \times 10^6$  cells per 100mm dish.

Media was removed and cells were washed by adding 10ml PBS, taking  
30 care not to disturb the cell layer. The dish was gently swirled and the PBS was immediately removed. PBS was  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free, which means it will dissociate the cells if left on too long. 2ml 0.04% Trypsin EDTA was added (for 100mm dish; 0.5 ml per well of 6-well dish; 4 drops per well of 24-well dish) to

the center of the dish and the dish was rotated to distribute the trypsin over the cell layer. Cells were incubated for 1-2 min. The dish was then tapped to dislodge the cells. Longer incubations were used if cells did not float free. Generally cells were not exposed to trypsin for more than a couple of minutes, as the trypsin tends to cause cell lysis if left on too long.

A new feeder was prepared by removing old media, washing with PBS as described above and then adding 15ml SCML. Each time the cells were exposed to trypsin was considered a passage.

Once the cells were no longer attached, 8ml SCML was added to the trypsin cell suspension and pipetted up and down vigorously to dissociate the cells (addition of the SCML inactivated the trypsin). 1ml cell suspension was transferred into each previously prepared feeder dishes.

Freezing of ES cells was conducted as follows. The ES cell suspension described above obtained after trypsinization was transferred into a 15 ml tube, and the cells were pelleted by centrifugation at 1000 rpm for 5 min. The supernatant was removed, taking care not to disturb the pellet. Usually a 100mm dish yielded enough cells to freeze 4 vials (approximately  $2-3 \times 10^6$  cells/vial). 1ml of freezing medium (50% FBS; 10% DMSO; 40% SCML) /vial was added and pipetted up and down to obtain a single cell suspension. 1ml cell suspension was placed in each cryovial, and cap tightened to obtain a tight seal. Freezing data was recorded in a data book. Cryovials were initially frozen in a -80°C freezer. The next day vials of frozen cells were transferred to a -150°C freezer for long-term storage.

ES cells were removed from feeders layers as follows. When cells were ready to be split, old media was removed and cells were washed with PBS, taking care not to disturb the colonies. The PBS wash was removed and 2ml trypsin was added (the trypsin was fresh and warm). The dish was immediately examined under a microscope. The dish was tapped during examination at 100X to dislodge the colonies. Only colonies with the correct ES cell morphology tended to come off easily. When the feeder layer started to pull loose, the dish was taken to a sterile hood. This process generally took about 30 seconds. In the hood, the dish was tilted so the trypsin and colonies pooled at

the lowest point, and 8ml SCML was added down the slope of the dish, followed by aspiration of the entire colony suspension back into the pipette.

The colony was transferred into a 15ml tube and pipetted up and down to dissociate the colonies into smaller clumps. Cells were pelleted by centrifugation at 1000 RPM for 5 minutes. The supernatant was aspirated and the cells were resuspended in 15 ml SCML and plated in a tissue culture treated dish without feeders. For cells that are not DBA-252 ES cells it is best to use gelatin coated dishes. ES cells from other mouse strains do not attach to plastic very well and work better with the matrix gelatin.

From this point on the cells were considered to be without feeders and after another passage were ready for in vitro differentiation. The standard maintenance protocol (above) was followed, but without feeders.

These methods are also described in M. Roach and J. McNeish (2002) Methods Mol. Biol. 185:1.

#### Example 2: Generation of Hepatocyte-like Cells from Embryonic Stem Cells

This example describes the differentiation of mouse ES cells into hepatocyte-like cells. The differentiation is described as comprising several steps: an embryoid body stage (days 0-5) during which embryoid bodies are formed; an early stage (days 6-8) during which the embryoid bodies were dissociated into a single cell suspension; a middle stage (days 9-11) during which cells formed a monolayer about 60-70% confluent; and a late stage (days 12-14) during which the cells were generally flatter, more epithelial-like in morphology and 60-70% confluent. Although a highly enriched population of hepatocyte-like cells was obtained at this stage, the differentiated cells were taken through a maturation and selection stage (days 15-18) during which non-hepatocyte-like cells are killed.

The cells were cultured as follows during the embryoid body stage (day 0-5). ES DBA252 cells were used in this Example. The preparation of these cells is described in Roach et al. (1995) Exp. Cell Res. 221:520. The ES cells used for *in vitro* differentiation were grown without primary embryonic fibroblast (PEF) feeders in the stem cell media (SCML) that contains 1000u/ml of leukemia inhibitory factor (LIF). When feeding feeder free ES cells care was taken so that ES cell colonies were not washed off. Two hours prior to dissociation, old media was removed and fresh SCML was added. After feeding, the media was

removed and the cells were washed with phosphate buffered saline (PBS) that does not contain calcium and magnesium (CMF). The PBS wash was removed, and 0.05% trypsin EDTA was added, followed by incubation for 1-2 minutes. The dish was tapped at 30-second intervals to dislodge the cells. When the cells  
5 were free floating in clumps, the trypsin was neutralized with equal volumes of SCML and the mixture was pipetted up and down to generate a single cell suspension.

When the cells were completely dissociated, an aliquot was removed to count, and the remaining cell suspension was pelleted by centrifugation at  
10 1000rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in SCML. The centrifugation and resuspension were repeated to ensure that all the trypsin was removed. After the second SCML wash, the cells were resuspended in 20ml HepEB media and plated in 2 x 100mm bacteriology dishes at  $1.5 \times 10^5$  cells/ml with a total volume of 10ml/dish. HepEB media  
15 consists of 80% Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO #31980-030); 5% PFHM-II (Protein-Free Hybridoma Medium) (GIBCO # 12040-077); 15% FBS (ES-Qualified Fetal Bovine Serum) (individual lots tested); 2mM L-Glutamine (GIBCO #25030-081);  $4 \times 10^{-4}$  Monothioglycerol (Sigma #M-6145); 50µg/ml L-Ascorbic Acid (A-4034); 300µg/ml Transferrin (GIBCO #13008-016);  
20 and 25ng/ml Gentamycin (GIBCO #15710-064). The cells were placed in a designated incubator. This was considered day zero (d0) of the experiment.

On days 2 and 4 the embryoid bodies (EBs) were fed. The EB suspension was transferred from both dishes into a 50ml tube and set aside. 5ml HepEB media was added to each dish and the dishes were returned to the  
25 incubator. The EBs in the 50ml tube settled to the bottom of the tube within about 10 minutes. When sufficient EBs pooled at the bottom, the supernatant was removed by aspiration and 10ml HepEB was added. 5ml of the EB suspension was transferred to the original 2 dishes and returned to the incubator. On day 5 the EB suspension was transferred from the 2 x 100mm  
30 bacteriology dishes into 3 x 100mm Collagen I coated dishes. An additional 5ml HepEB media was added to each dish so that the total volume was 15ml per dish.

The cells were cultured as follows during the early stage (day 6-8). On day 6 the HepEB media was removed and 15ml HepI media was added. HepI media consists of 80% Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO #31980-030); 5% PFHM-II (Protein-Free Hybridoma Medium) (GIBCO # 12040-077); 15% FBS (ES-Qualified Fetal Bovine Serum) (individual lots tested); 2mM L-Glutamine (GIBCO #25030-081);  $4 \times 10^{-4}$  Monothioglycerol (Sigma #M-6145); 50 $\mu$ g/ml L-Ascorbic Acid (A-4034); 10 $\mu$ M Nicotinamide (Sigma #N-0636); 10ng/ml EGF murine recombinant (BD #354001); 10ng/ml Acidic FGF (human recombinant fibroblast growth factor (GIBCO #13241-013); and 25ng/ml Gentamycin (GIBCO #15710-064). The EBs were attached to the collagen I matrix and had begun to spread out.

On day 7 the old media was removed and 15ml fresh HepI media was added. The EBs were very spread and some were touching each other. On day 8, the old media was removed and cells were washed with 10ml PBS/CMF. The PBS was removed and 2ml 0.05% trypsin EDTA added, followed by an incubation for 1-2 minutes, while tapping the dish at 30-second intervals. When cells and EB clumps were free floating, the trypsin was neutralized with 8ml HepI and the mixture was pipetted up and down to dissociate the cells into a single cell suspension. 10ml of cell suspension was transferred to a conical tube (all dishes for each cell line were pooled together). Cells were counted and then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cells were plated at  $2 \times 10^6$  cells per 100mm Collagen I dish in HepI with a total volume of 15ml. At least 4 x 100mm dishes were plated per cell line. The remaining cells were frozen in HepI freezing media.

The cells were cultured as follows during the middle stage (days 9-11). On days 9 and 10, cells were examined and observations recorded. On day 9, the HepI media was removed and the cells were fed with 15ml HepII media. HepII media consisted of 80% Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO #31980-030); 5% PFHM-II (Protein-Free Hybridoma Medium) (GIBCO # 12040-077); 15% FBS (ES-Qualified Fetal Bovine Serum) (individual lots tested); 2mM L-Glutamine (GIBCO #25030-081);  $4 \times 10^{-4}$  Monothioglycerol (Sigma #M-6145); 0.1mM MEM Non-Essential Amino Acids (GIBCO #11140-050); 10 $\mu$ M Nicotinamide (Sigma #N-0636); 10ng/ml EGF murine recombinant epidermal

growth factor (BD #354001); 10ng/ml Acidic FGF human recombinant fibroblast growth factor (GIBCO #13241-013); 25ng/ml HGF/SF human recombinant hepatocyte growth factor (BD #354103); and 25ng/ml Gentamycin (GIBCO #15710-064). Cells formed a monolayer, around 60-70% confluent.

- 5 On day 11, the HepII was removed and the cells were washed with 10ml PBS. PBS was removed and 2ml 0.04% trypsin EDTA added, followed by an incubation for 1-2 minutes. When cells became free-floating (assisted by tapping the dish), 8ml HepII was added to neutralize the trypsin, and the solution was pipetted up and down to dissociate the cells into a single cell suspension for  
10 transfer into a conical tube. Cells were counted and then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and cells were plated  $2 \times 10^6$  cells per 100mm Collagen I dish and at least 4 dishes were plated. The remaining cells were frozen in HepII freezing media.

- The cells were cultured as follows during the late stage (days 12-14). On  
15 days 12 and 13, the cells were examined and observations recorded. On day 12, the old media was removed and cells were fed with 15ml HepIII media per 100mm dish. HepIII media consisted of 80% Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO #31980-030); 5% PFHM-II (Protein-Free Hybridoma Medium) (GIBCO # 12040-077); 15% FBS (ES-Qualified Fetal Bovine Serum)  
20 (individual lots tested); 2mM L-Glutamine (GIBCO #25030-081);  $4 \times 10^{-4}$  Monothioglycerol (Sigma #M-6145); 0.1mM MEM Non-Essential Amino Acids (GIBCO #11140-050); 10 $\mu$ M Nicotinamide (Sigma #N-0636); 10ng/ml EGF murine recombinant epidermal growth factor (BD #354001); 25ng/ml HGF/SF human recombinant hepatocyte growth factor (BD #354103); 10ng/ml OSM  
25 murine oncostatin M (R&D Systems #495-MO-025); 100nM Dexamethasone (Sigma #D-8893); 1x ITS insulin-transferrin-selenium-G (insulin 10  $\mu$ g/ml, transferrin 5  $\mu$ g/ml, selenium 5 ng/ml; GIBCO #41400-045); and 25ng/ml Gentamycin (GIBCO #15710-064). Cells were generally flatter, more epithelial-like in morphology and 60-70% confluent.

- 30 On day 14, old media was removed, and the cells were washed with 10ml PBS. 2ml 0.05% trypsin was added followed by an incubation for 1-2 minutes. When cells became free-floating (after tapping the dish) 8ml HepIII was added and pipetted up and down to dissociate into a single cell suspension for transfer



into a conical tube. Cells were counted and centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and plated at  $3 \times 10^6$  cells per 100mm collagen I dish, and  $2 \times 10^5$  cells per well in a 24-well collagen I dish in HepIII. As many 24-well dishes were plated as needed for assays.

5        The cells were cultured as follows during the maturation and selection stage (days 15-18), i.e., stage during which the hepatocyte-like cells are enriched. On days 15 and 16, the cells were examined and observations were recorded. Old media was removed and 15ml HepIV medium added. Hep IV medium consisted of 90% DMEM without glucose (GIBCO #11966-025); 10%  
10    FBS (ES-Qualified Fetal Bovine Serum) (GIBCO #10439-024); 2mM L-Glutamine (GIBCO #25030-081);  $4 \times 10^{-4}$  Monothioglycerol (Sigma #M-6145); 0.1mM MEM Non-Essential Amino Acids (GIBCO #11140-050); 10 $\mu$ M Nicotinamide (Sigma #N-0636); 1mM Pyruvic Acid (P-4562); 10ng/ml EGF murine recombinant epidermal growth factor (BD #354001); 25ng/ml HGF/SF  
15    human recombinant hepatocyte growth factor (BD #354103); 10ng/ml OSM murine oncostatin M (R&D Systems #495-MO-025); 100nM Dexamethasone (Sigma #D-8893); 25ng/ml Gentamycin (GIBCO #15710-064); and 5mM Sodium Butyric Acid (B-5887). At this stage, cells began to look like they were dying. The glucose-free media selects for cells that are capable of undergoing  
20    gluconeogenesis using pyruvate as the substrate. The sodium butyrate will generally not be detrimental to hepatocytes but will generally be lethal to other cell types.

On day 17 and 18 there was much cell debris from cell death due to selection in glucose-free and sodium butyrate media. On day 17, the old media  
25    was removed and cells were washed with 10ml PBS. Following the PBS wash, cells were fed with 15ml HepIV. On day 19 and 20, cells were examined and observations were recorded. Cells were washed with 10ml PBS then fed 15ml HepIII. Cell morphology was generally very flat and cuboidal in shape. At this stage, the cells are ready to use in assays or, to improve metabolism, induction  
30    agents can be used prior to assays.

For induction of hepatic metabolism, old media was removed and Hep III that contains 100nM Pregnenolone 16 $\alpha$ -carbonitrile was added. The next day old media was removed and fresh Hep V was added. Hep V medium consisted

of 90% Williams Media E (GIBCO #12551-032); 10% FBS (ES-Qualified Fetal Bovine Serum) (GIBCO #10439-024); 2mM L-Glutamine (GIBCO #25030-081);  $4 \times 10^{-4}$  Monothioglycerol (Sigma #M-6145); 10 $\mu$ M Nicotinamide (Sigma #N-0636); 10ng/ml OSM murine oncostatin M (R&D Systems #495-MO-025); 100nM  
5 Dexamethasone (Sigma #D-8893); 1x ITS insulin-transferrin-selenium-G (GIBCO #41400-045); and 25ng/ml Gentamycin (GIBCO #15710-064).

Example 3: Hepatopoietin stimulates proliferation of cells differentiating from ES cells into hepatocyte-like cells

This Example describes that the addition of mouse hepatopoietin during  
10 the middle, late and/or maturation and selection stages stimulates the growth of the cells that are differentiating into hepatocyte-like cells.

Mouse ES cells were differentiated into hepatocyte-like cells as described above, with the addition of 50ng/ml mouse hepatopoietin protein to the HepII, HepIII and/or HepIV media. The mouse hepatopoietin protein consisted of a  
15 portion of the wild-type protein, which portion has the amino acid sequence set forth in SEQ ID NO: 16.

A nucleic acid encoding the protein was obtained as follows. Two primers were synthesized based on the nucleotide sequence of mouse augments of liver regeneration (Alr) mRNA set forth in GenBank Accession number  
20 AF148688 (SEQ ID NO: 15). The 5' primer used had the sequence 5' *tattcat*ATGCGGACCCAGCAGAAGCGGGACAT 3' (SEQ ID NO: 23) and consisted of an HPO sequences (indicated in large caps) linked 5' to an NdeI site (indicated in italics). The 3' primer used had the sequence 5' *tatca*CTAGTCACAGGAGCCGTCCTTCCAT 3' (SEQ ID NO: 24) and consisted of  
25 an HPO sequence (indicated in large caps) linked 5' to two stop codons. These primers were used to amplify mouse HPO from mouse liver cDNA. Since the clone obtained from this amplification contained a mutation at the 3' end relative to the sequence in GenBank accession number AF148688, this clone was amplified again using the same 5' end primer described above and the following  
30 3' end primer: 5' TCACTAGTCACAGGAGCCGTCCTTCCATCCGT 3' (SEQ ID NO: 25). This PCR fragment was subsequently cloned into pCR 2.1 TOPO vector according to the manufacturer's protocol. Several hundred white colonies were retrieved from Amp/LB plates. Three clones (#1-3) were sequenced and a

sequence alignment with AF148688 indicated that each clone contained the expected sequence.

The final clone contained the following insert:

5' CATATGCGGACCCAGCAGAAGCGGGACATCAAGTTTAGGGAGGACTGT  
**CCGCAGGATCGGGAAGAATTGGGTGCGCCACACCTGGGCTTTCCTCCATAC**  
**GCTGGCCGCCTATTACCCGGACAGGCCACGCCAGAACAACAACAGGAT**  
**ATGGCCCAGTTCATACATATATTTTCCAAGTTTTACCCCTGCGAGGAATGT**  
**GCGGAAGACATAAGGAAGAGGATAGGCAGGAACCAGCCAGACACAAGC**  
**ACTCGAGTATCCTTCAGCCAGTGGCTGTGCCGCCTGCACAATGAGGTGAA**  
10 **TCGGAAGCTGGGCAAGCCTGATTTGACTGCTCGAGAGTAGATGAGCGTT**  
**GGCGTGACGGATGGAAGGACGGCTCCTGTGACTAGTGAAAGGGCGAATT**  
**CTGCAGATATCCATCACACTGGCGGCCGC** (SEQ ID NO: 26).

The NdeI site (CATATG) and the NotI site (GCGGCCGC) are underlined. The HPO coding sequence is indicated in bold and codes for the following 125 amino acid protein:

15 MRTQQKRDIKFREDCPQDREELGRHTWAFLHTLAAYYPDRPTPEQQQDMAQ  
 FIHIFSKFYPCEECAEDIRKRIGRNQPDSTRVSFSQWLCRLHNEVNRKLGKPD  
 FDCSRVDERWRDGWKDGSCD (SEQ ID NO:16).

The purified hepatopoietin protein used in the in vitro differentiation  
 20 cultures was prepared as follows. The insert of the above-described clone was subcloned into pET23b(+) (Novagen) between the NdeI and NotI sites. This construct was named pMCG204. pMCG204 was transformed into BL21(gold)DE3 cells (Stratagene) and Origami(DE3) cells (Novagen) pursuant to the manufacturer's instructions. Single colonies were inoculated in 25 ml 2xYT  
 25 media with 100  $\mu$ g/ml carbenicillin for BL21(gold)DE3 strain clones or 2xYT media with 100  $\mu$ g/ml carbenicillin, 15  $\mu$ g/ml kanamycin, and 12.5  $\mu$ g/ml tetracycline for Origami(DE3) strain clones. Cultures were grown overnight at 37°C with shaking. 23 ml of the overnight culture was used to inoculate 1 liter of LB broth with appropriate antibiotic(s) in 2.8 liter tri-baffled Fernbach flasks.  
 30 These were grown at 37°C with shaking until the O.D. 600 reached 0.75 and then induced with IPTG to a final concentration of 0.6mM. Growth was continued overnight at 37°C and cell pellets were harvested the next day.

The cell pellets from either strain were resuspended in 50 mM NaAcetate, pH 5.2, 5 mM DTT, and 1 tablet Complete-EDTA protease inhibitor (Roche) per

25 ml buffer. Lysis was achieved by sonication with 12 ml buffer per 1 liter of cell paste. The cell lysate was then spun at 15,000 rpm in a Sorvall RC 5B plus centrifuge in an SS34 rotor for 20 minutes at 4°C. Supernatant was applied to a 1 ml HiTrap SP XL column (Pharmacia) equilibrated with buffer A (50 mM NaAcetate, pH5.2, 5 mM DTT). The column was washed with several column volumes of buffer A and protein was eluted with a gradient from 0 to 100% buffer B (buffer A with 1 M NaCl) over 25 ml.

Fractions containing soluble HPO were pooled and concentrated with a Centriplus 3,000 NMWCO membrane device. The concentrate was applied to a size exclusion chromatography on Superdex 75 prep grade HiLoad 16/60 column (Pharmacia) previously equilibrated with PBS (Gibco Catalog #: 14190-136). Fractions containing HPO were pooled.

The results of the in vitro differentiation of ES cells with the presence of mouse HPO in the HepII, HepII and/or HepIV media indicate that the presence of HPO stimulates the proliferation of cells giving rise to hepatocyte-like cells, thereby resulting in populations of cells having a higher percentage of hepatocyte-like cells. The presence of hepatopoietin in either medium had this effect, but the strongest effect was seen when it was included in all three media, i.e., during the middle stage, the late stage and the maturation and selection stage. In this case, it appeared that over 90% of the cells in the culture were hepatocyte-like cells.

Example 4: Phenotypic characteristics of hepatocyte-like cells and precursors thereof

The expression of several genes was monitored during the differentiation of ES cells into hepatocyte-like cells. The genes included  $\alpha$ -fetoprotein;  $\gamma$ -glutaryltransferase; hepatocyte nuclear factor (HNF) 1 $\alpha$ ; HNF 1 $\beta$ ; HNF 3 $\alpha$ ; HNF 3 $\beta$ ; HNF 4; albumin; anti-trypsin; transthyretin and cystic fibrosis transmembrane conductance regulator (CFTR).

The level of expression of these genes was monitored by quantitative reverse transcription polymerase chain reaction (RT-PCR) using the primers set forth in Table II:

**Table II.** PCR primers for hepatocyte-specific markers (GIBCO-BRL)

Marker	Forward Primer	Reverse Primer
$\alpha$ -Fetoprotein	CAGCCAAAGTGGAGTGGAAAG A (SEQ ID NO:27)	AACTCTCGGCAGGTTCTGGAA (SEQ ID NO:28)
$\gamma$ -Glutaryl- transferase	ATTGAGAAGACCCCTGCCTTGT (SEQ ID NO:29)	ATCTGCAATGTGTCTAGCCAGC (SEQ ID NO:30)
HNF1 $\alpha$	ATTGAGAAGACCCCTGCCTTGT (SEQ ID NO:31)	ATCTGCAATGTGTCTAGCCAGC (SEQ ID NO:32)
HNF1 $\beta$	CCTGAACCAATCCCACCTCTCT (SEQ ID NO:33)	ATCTCCCGTTGCTTTCTGACG (SEQ ID NO:34)
HNF3 $\alpha$	ATTGAGAAGACCCCTGCCTTGT (SEQ ID NO:35)	ATCTGCAATGTGTCTAGCCAGC (SEQ ID NO:36)
HNF3 $\beta$	AAGAAGATGGCTTTCAGGCC (SEQ ID NO:37)	AAGGCCATTGAAGTGTGGTGG (SEQ ID NO:38)
HNF4	GACTCTCTAAAACCCTTGCCGG (SEQ ID NO:39)	CCATGGTCAACACCTGCACAT (SEQ ID NO:40)
Albumin	CGCCCATCGGTATAATGATTG (SEQ ID NO:41)	CTGCACTAATTGGCATGCTCA (SEQ ID NO:42)
Anti-Trypsin	TGCTTGATGTGCACCATTGC (SEQ ID NO:43)	TGCTCCAGATGCTGCATCTTC (SEQ ID NO:44)
Transthyreti n	AAGCAGAGTGGACCAACCGTT (SEQ ID NO:45)	AAGCAGAGTGGACCAACCGTT (SEQ ID NO:46)
CFTR	TTAATGTGCTTGGCCCGATC (SEQ ID NO:47)	CCAGCGAAGGCTTGTTTTAGAA (SEQ ID NO:48)

The results are set forth in Table III (wherein "X" represents that expression was detected), and show that some genes are expressed from day 3 to day 29 of the culture, whereas others are expressed specifically at certain stages of differentiation, as expected.

**Table III.** RT-PCR results for ES cell-derived hepatocytes

Markers	Days of <i>In Vitro</i> Differentiation																
	3	4	5	6	7	8	9	10	11	12	15	17	19	22	24	26	29
$\alpha$ -fetoprotein	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
$\gamma$ -glutaryl- transferase	x	x	x	x	x	x	x	x	x	x					x		x
HNF 1 $\alpha$	x	x	x	x	x	x	x	x	x	x					x		x
HNF 1 $\beta$	x	x	x	x	x	x	x	x	x	x	x		x		x		x
HNF 3 $\alpha$	x	x	x	x	x	x	x	x	x	x					x		x
HNF 3 $\beta$	x	x	x	x	x	x	x	x	x	x	x		x		x		x
HNF 4	x	x	x	x	x	x	x	x	x	x	x						x
Albumin						x	x	x	x	x							
Anti-trypsin						x	x	x	x	x							x
Transthyretin	x		x	x	x	x	x	x	x	x							x
CFTR	x					x	x	x	x	x							x

To further characterize the hepatocyte-like cells, cytochrome p450 proteins were detected by immunohistochemical detection. Differentiated hepatocyte-like cells

were fixed with 4% paraformaldehyde, treated with specific goat anti-rat CYP sera recognizing CYP1A1, CYP2B1, CYP2C6, CYP2C11, CYP2C13, CYP3A2 or CYP4A1 (Daiichi Pure Chemical Co. LTD, Tokyo, Japan), washed, and the treated with an alkaline phosphatase-labeled rabbit anti-goat antibody, according to methods known in the art. Labeled cells were detected with the alkaline phosphatase substrate NBT/BCIP. After treatment, cells were counterstained with eosin. The results indicate the presence of all of the CYPs tested, with higher levels of CYP1A1, CYP2B1 and CYP2C6, as is seen in primary cultures of differentiated hepatocytes. Thus, these results confirm that the differentiated cells obtained from ES cells have characteristics of differentiated hepatocytes.

Example 5: Functional characteristics of hepatocyte-like cells

The presence of CYP proteins in the hepatocyte-like cells was also determined by monitoring the conversion of certain compounds added to the hepatocyte-like cells. Two test compounds (7-ethoxy-coumarin and dextromethorphan) were added to hepatocyte-like cells. The supernatants of the cells were then subjected to high pressure liquid chromatography (HPLC) and mass spectrometry to identify products of the conversion of the test compounds by CYP3a, CYP2d, CYP2e1 and CYP1a2. The expected conversion products were obtained in each case, thereby indicating that these cytochrome p450 enzymes are present and functionally active in the hepatocyte-like cells.

Another test that was used to characterize the hepatocyte-like cells is the dibenzylfluorescein (DBF) assay for metabolic activity. See, for example, Stresser et al., *Drug Metab. Disp.*, 28:1440-48 (2000). These assays were conducted using a DBF compound (Molecular Probes, Eugene OR) according to the manufacturer's recommendations. The results indicate that the test was positive, and that similar results were obtained with hepatocyte-like cells obtained from differentiation in the presence or in the absence of HPO.

EQUIVALENTS

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The appended claims should be interpreted by

reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

All publications and patents mentioned herein, including those items listed below, are hereby incorporated by reference in their entirety as if each individual  
5 publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

CLAIMS:

1. A method for obtaining a hepatocyte-like cell, comprising:
  - a) providing a stem cell;
  - 5 b) culturing the stem cell in a first medium comprising effective amounts of an acidic fibroblast growth factor (aFGF) and an epidermal growth factor (EGF) for about 2 to 4 days to obtain a first cell population;
  - c) culturing a cell of the first cell population in a second medium comprising an effective amount of hepatocyte growth factor (HGF) for about 2 to 4 days to obtain a second cell population; and
  - 10 d) culturing a cell of the second cell population in a third medium comprising effective amounts of oncostatin-M for about 2 to 4 days to obtain a third cell population, the third cell population comprising a plurality of hepatocyte-like cells.
- 15 2. The method of claim 1, wherein the aFGF is present at a concentration of about 1-20 ng/ml and the EGF is present at a concentration of about 1-20 ng/ml in the first medium.
- 20 3. The method of claim 1, wherein the HGF is present at a concentration of about 5-50 ng/ml in the second medium.
4. The method of claim 1, wherein the oncostatin-M is present at a concentration of about 1-30 ng/ml in the third medium.
- 25 5. The method of claim 1, further comprising:
  - e) culturing a plurality of cells of the third cell population in a medium suitable for selectively culturing gluconeogenic cells, thereby obtaining a cellular composition comprising an enriched population of
  - 30 hepatocyte-like cells.
6. The method of claim 5, further comprising:
  - f) culturing a plurality of cells of the enriched population of hepatocyte-like cells in a medium suitable for stimulating hepatocyte-



associated metabolic functions, thereby obtaining a cellular composition comprising a population of hepatocyte-like cells having enhanced hepatocyte-associated metabolic activity.

5           7.     The method of claim 6, further comprising:

g) culturing a hepatocyte-like cell in a fourth medium comprising nicotinamide, oncostatin M, dexamethasone, insulin, transferrin and selenium.

10          8.     The method of claim 1, further comprising:

e) culturing a plurality of cells of the third cell population in a medium suitable for inducing hepatocyte-like metabolic functions, thereby obtaining a cellular composition comprising a population of hepatocyte-like cells having enhanced hepatocyte-like metabolic activity.

15

9.     A method for obtaining a hepatocyte-like cell, comprising:

a)     providing an ES cell;

b)     stimulating the differentiation of the ES cell into embryoid bodies for about 5 days;

20          c)     culturing the embryoid bodies in a first medium comprising effective amounts of an aFGF and an EGF for about 1 to 2 days to obtain embryoid bodies;

25          d)     dissociating the embryoid bodies into a single cell suspension and culturing the single cell suspension for about 1 to 2 days in the first medium to obtain a first cell population;

e)     culturing a cell of the first cell population in a second medium comprising an effective amount of EGF, HGF and aFGF for about 2 to 4 days to obtain a second cell population; and

30          f)     culturing a cell of the second cell population in a third medium comprising effective amounts of oncostatin-M, EGF, and HGF for about 2 to 4 days to obtain a third cell population, the third cell population comprising a plurality of hepatocyte-like cells.

10. A cellular composition comprising viable cells, wherein at least 90% of the viable cells are hepatocyte-like cells.
11. The cellular composition of claim 10, wherein the hepatocyte-like  
5 cells:
- a) use pyruvate as a carbon source
  - b) express two or more cytochrome P450 enzymes; and
  - c) are viable in 5mM butyric acid.
- 10 12. A cellular composition obtained by the method of claim 1 or 9.
13. A method for treating a subject in need of liver cells, comprising administering to the subject a therapeutically effective amount of the hepatocyte-like cells of claim 12.
- 15 14. An isolated nucleic acid encoding a polypeptide having SEQ ID NO: 18.
- 15 15. An isolated polypeptide comprising the amino acid sequence SEQ  
20 ID NO: 18.

## SEQUENCE LISTING

&lt;110&gt; PFIZER PRODUCTS INC.

&lt;120&gt; GROWTH AND DIFFERENTIATION OF STEM CELLS

&lt;130&gt; PC25028A

&lt;150&gt; US 60/459,449

&lt;151&gt; 2003-03-31

&lt;160&gt; 48

&lt;170&gt; PatentIn version 3.2

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His Pro Arg Ala Arg Arg Leu Phe Trp Thr Asp Val Gly Met Ser Pro  
610 615 620

Arg Ile Glu Ser Ala Ser Leu Gln Gly Ser Asp Arg Val Leu Ile Ala  
625 630 635 640

Ser Ser Asn Leu Leu Glu Pro Ser Gly Ile Thr Ile Asp Tyr Leu Thr  
645 650 655

Asp Thr Leu Tyr Trp Cys Asp Thr Lys Arg Ser Val Ile Glu Met Ala  
660 665 670

Asn Leu Asp Gly Ser Lys Arg Arg Arg Leu Ile Gln Asn Asp Val Gly  
675 680 685

His Pro Phe Ser Leu Ala Val Phe Glu Asp His Leu Trp Val Ser Asp  
690 695 700

Trp Ala Ile Pro Ser Val Ile Arg Val Asn Lys Arg Thr Gly Gln Asn  
705 710 715 720

Arg Val Arg Leu Gln Gly Ser Met Leu Lys Pro Ser Ser Leu Val Val  
725 730 735

Val His Pro Leu Ala Lys Pro Gly Ala Asp Pro Cys Leu Tyr Arg Asn  
740 745 750

Gly Gly Cys Glu His Ile Cys Gln Glu Ser Leu Gly Thr Ala Arg Cys  
755 760 765

Leu Cys Arg Glu Gly Phe Val Lys Ala Trp Asp Gly Lys Met Cys Leu  
770 775 780

Pro Gln Asp Tyr Pro Ile Leu Ser Gly Glu Asn Ala Asp Leu Ser Lys  
 785 790 795 800  
 Glu Val Thr Ser Leu Ser Asn Ser Thr Gln Ala Glu Val Pro Asp Asp  
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 Asp Gly Thr Glu Ser Ser Thr Leu Val Ala Glu Ile Met Val Ser Gly  
 820 825 830  
 Met Asn Tyr Glu Asp Asp Cys Gly Pro Gly Gly Cys Gly Ser His Ala  
 835 840 845  
 Arg Cys Val Ser Asp Gly Glu Thr Ala Glu Cys Gln Cys Leu Lys Gly  
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 Phe Ala Arg Asp Gly Asn Leu Cys Ser Asp Ile Asp Glu Cys Val Leu  
 865 870 875 880  
 Ala Arg Ser Asp Cys Pro Ser Thr Ser Ser Arg Cys Ile Asn Thr Glu  
 885 890 895  
 Gly Gly Tyr Val Cys Arg Cys Ser Glu Gly Tyr Glu Gly Asp Gly Ile  
 900 905 910  
 Ser Cys Phe Asp Ile Asp Glu Cys Gln Arg Gly Ala His Asn Cys Ala  
 915 920 925  
 Glu Asn Ala Ala Cys Thr Asn Thr Glu Gly Gly Tyr Asn Cys Thr Cys  
 930 935 940  
 Ala Gly Arg Pro Ser Ser Pro Gly Arg Ser Cys Pro Asp Ser Thr Ala  
 945 950 955 960  
 Pro Ser Leu Leu Gly Glu Asp Gly His His Leu Asp Arg Asn Ser Tyr  
 965 970 975  
 Pro Gly Cys Pro Ser Ser Tyr Asp Gly Tyr Cys Leu Asn Gly Gly Val  
 980 985 990  
 Cys Met His Ile Glu Ser Leu Asp Ser Tyr Thr Cys Asn Cys Val Ile  
 995 1000 1005  
 Gly Tyr Ser Gly Asp Arg Cys Gln Thr Arg Asp Leu Arg Trp Trp  
 1010 1015 1020  
 Glu Leu Arg His Ala Gly Tyr Gly Gln Lys His Asp Ile Met Val  
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 Val Ala Val Cys Met Val Ala Leu Val Leu Leu Leu Leu Leu Gly  
 1040 1045 1050

Met Trp Gly Thr Tyr Tyr Tyr Arg Thr Arg Lys Gln Leu Ser Asn  
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Pro Pro Lys Asn Pro Cys Asp Glu Pro Ser Gly Ser Val Ser Ser  
 1070 1075 1080

Ser Gly Pro Asp Ser Ser Ser Gly Ala Ala Val Ala Ser Cys Pro  
 1085 1090 1095

Gln Pro Trp Phe Val Val Leu Glu Lys His Gln Asp Pro Lys Asn  
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Gly Ser Leu Pro Ala Asp Gly Thr Asn Gly Ala Val Val Asp Ala  
 1115 1120 1125

Gly Leu Ser Pro Ser Leu Gln Leu Gly Ser Val His Leu Thr Ser  
 1130 1135 1140

Trp Arg Gln Lys Pro His Ile Asp Gly Met Gly Thr Gly Gln Ser  
 1145 1150 1155

Cys Trp Ile Pro Pro Ser Ser Asp Arg Gly Pro Gln Glu Ile Glu  
 1160 1165 1170

Gly Asn Ser His Leu Pro Ser Tyr Arg Pro Val Gly Pro Glu Lys  
 1175 1180 1185

Leu His Ser Leu Gln Ser Ala Asn Gly Ser Cys His Glu Arg Ala  
 1190 1195 1200

Pro Asp Leu Pro Arg Gln Thr Glu Pro Val Lys  
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 <213> Homo sapiens

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 gaagaaatac aattcatgaa ttcaaaaaat cagcaaagac taccctaatac aaaatagatc 240  
 cagcactgaa gataaaaacc aaaaaagtga atactgcaga ccaatgtgct aatagatgta 300  
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 aatttgacct ctatgaaaac aaagactaca ttagaaactg catcattggg aaaggacgca 480

gctacaaggg aacagtatct atcactaaga gtggcatcaa atgtcagccc tggagttcca 540  
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 accctcacac ccgctgggag tactgtgcaa ttaaaacatg cgagacataa catgggctct 960  
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20 25 30

Arg Lys Arg Arg Asn Thr Ile His Glu Phe Lys Lys Ser Ala Lys Thr  
35 40 45

Thr Leu Ile Lys Ile Asp Pro Ala Leu Lys Ile Lys Thr Lys Lys Val  
50 55 60

Asn Thr Ala Asp Gln Cys Ala Asn Arg Cys Thr Arg Asn Lys Gly Leu  
65 70 75 80

Pro Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ala Arg Lys Gln Cys  
85 90 95

Leu Trp Phe Pro Phe Asn Ser Met Ser Ser Gly Val Lys Lys Glu Phe  
100 105 110

Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg Asn Cys  
115 120 125

Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr Lys  
 130 135 140  
 Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser Met Ile Pro His Glu His  
 145 150 155 160  
 Ser Phe Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn Tyr  
 165 170 175  
 Cys Arg Asn Pro Arg Gly Glu Glu Gly Gly Pro Trp Cys Phe Thr Ser  
 180 185 190  
 Asn Pro Glu Val Arg Tyr Glu Val Cys Asp Ile Pro Gln Cys Ser Glu  
 195 200 205  
 Val Glu Cys Met Thr Cys Asn Gly Glu Ser Tyr Arg Gly Leu Met Asp  
 210 215 220  
 His Thr Glu Ser Gly Lys Ile Cys Gln Arg Trp Asp His Gln Thr Pro  
 225 230 235 240  
 His Arg His Lys Phe Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe Asp  
 245 250 255  
 Asp Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp Cys Tyr  
 260 265 270  
 Thr Leu Asp Pro His Thr Arg Trp Glu Tyr Cys Ala Ile Lys Thr Cys  
 275 280 285  
 Ala Asp Asn Thr Met Asn Asp Thr Asp Val Pro Leu Glu Thr Thr Glu  
 290 295 300  
 Cys Ile Gln Gly Gln Gly Glu Gly Tyr Arg Gly Thr Val Asn Thr Ile  
 305 310 315 320  
 Trp Asn Gly Ile Pro Cys Gln Arg Trp Asp Ser Gln Tyr Pro His Glu  
 325 330 335  
 His Asp Met Thr Pro Glu Asn Phe Lys Cys Lys Asp Leu Arg Glu Asn  
 340 345 350  
 Tyr Cys Arg Asn Pro Asp Gly Ser Glu Ser Pro Trp Cys Phe Thr Thr  
 355 360 365  
 Asp Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Asn Cys Asp  
 370 375 380  
 Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys Asn Tyr Met  
 385 390 395 400

Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr Cys Ser Met Trp Asp  
405 410 415

Lys Asn Met Glu Asp Leu His Arg His Ile Phe Trp Glu Pro Asp Ala  
420 425 430

Ser Lys Leu Asn Glu Asn Tyr Cys Arg Asn Pro Asp Asp Asp Ala His  
435 440 445

Gly Pro Trp Cys Tyr Thr Gly Asn Pro Leu Ile Pro Trp Asp Tyr Cys  
450 455 460

Pro Ile Ser Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val Asn Leu  
465 470 475 480

Asp His Pro Val Ile Ser Cys Ala Lys Thr Lys Gln Leu Arg Val Val  
485 490 495

Asn Gly Ile Pro Thr Arg Thr Asn Ile Gly Trp Met Val Ser Leu Arg  
500 505 510

Tyr Arg Asn Lys His Ile Cys Gly Gly Ser Leu Ile Lys Glu Ser Trp  
515 520 525

Val Leu Thr Ala Arg Gln Cys Phe Pro Ser Arg Asp Leu Lys Asp Tyr  
530 535 540

Glu Ala Trp Leu Gly Ile His Asp Val His Gly Arg Gly Asp Glu Lys  
545 550 555 560

Cys Lys Gln Val Leu Asn Val Ser Gln Leu Val Tyr Gly Pro Glu Gly  
565 570 575

Ser Asp Leu Val Leu Met Lys Leu Ala Arg Pro Ala Val Leu Asp Asp  
580 585 590

Phe Val Ser Thr Ile Asp Leu Pro Asn Tyr Gly Cys Thr Ile Pro Glu  
595 600 605

Lys Thr Ser Cys Ser Val Tyr Gly Trp Gly Tyr Thr Gly Leu Ile Asn  
610 615 620

Tyr Asp Gly Leu Leu Arg Val Ala His Leu Tyr Ile Met Gly Asn Glu  
625 630 635 640

Lys Cys Ser Gln His His Arg Gly Lys Val Thr Leu Asn Glu Ser Glu  
645 650 655

Ile Cys Ala Gly Ala Glu Lys Ile Gly Ser Gly Pro Cys Glu Gly Asp  
660 665 670

Tyr Gly Gly Pro Leu Val Cys Glu Gln His Lys Met Arg Met Val Leu  
675 680 685

Gly Val Ile Val Pro Gly Arg Gly Cys Ala Ile Pro Asn Arg Pro Gly  
690 695 700

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Leu Thr Tyr Lys Val Pro Gln Ser  
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<212> DNA  
<213> Mus musculus

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catgaattta aaaagtcagc aaaaactact cttaccaagg aagaccatt actgaagatt 180  
aaaacaaaa aagtgaactc tgcagatgag tgtgccaaca ggtgtatcag gaacaggggc 240  
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gaaaacaaag actatattag aaactgcac attggtaaag gaggcagcta taaagggacg 420  
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atgtggaatg gaattccctg tcagcgttgg gattcgcagt accctcaca gcatgatatc 1020  
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gctgaatcac catggtgttt taccactgac ccaaaccatc gagttggcta ctgctctcaa 1140  
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atgggcaact tatccaaaac aaggctctgga cttacatgtt ccatgtggga caagaatatg 1260  
gaggatttac accgtcatat cttctgggag ccagatgcta gcaaattgaa taagaattac 1320



tgccggaatc ctgatgatga tgcccatgga ccttggtgct acacggggaa tcctcttatt 1380  
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 ttggaccatc ctgtaatatc ctgtgccaaa acaaaacaac tgcgggttgt aaatggcatt 1500  
 ccaacacaaa caacagtagg gtggatgggt agtttgaaat acagaaataa acatatctgt 1560  
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 gatttaccta gttatggttg tacaatccct gaaaagacca cttgcagtat ttacggctgg 1860  
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 <212> PRT  
 <213> Mus musculus

<400> 12

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Leu His Leu Leu Leu Leu His Val Ala Ile Pro Tyr Ala Glu Gly Gln  
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Lys Lys Arg Arg Asn Thr Leu His Glu Phe Lys Lys Ser Ala Lys Thr  
35 40 45

Thr Leu Thr Lys Glu Asp Pro Leu Leu Lys Ile Lys Thr Lys Lys Val  
50 55 60

Asn Ser Ala Asp Glu Cys Ala Asn Arg Cys Ile Arg Asn Arg Gly Phe  
65 70 75 80

Thr Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ser Arg Lys Arg Cys  
85 90 95

Tyr Trp Tyr Pro Phe Asn Ser Met Ser Ser Gly Val Lys Lys Gly Phe  
100 105 110

Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg Asn Cys  
 115 120 125  
 Ile Ile Gly Lys Gly Gly Ser Tyr Lys Gly Thr Val Ser Ile Thr Lys  
 130 135 140  
 Ser Gly Ile Lys Cys Gln Pro Trp Asn Ser Met Ile Pro His Glu His  
 145 150 155 160  
 Ser Phe Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn Tyr  
 165 170 175  
 Cys Arg Asn Pro Arg Gly Glu Glu Gly Gly Pro Trp Cys Phe Thr Ser  
 180 185 190  
 Asn Pro Glu Val Arg Tyr Glu Val Cys Asp Ile Pro Gln Cys Ser Glu  
 195 200 205  
 Val Glu Cys Met Thr Cys Asn Gly Glu Ser Tyr Arg Gly Pro Met Asp  
 210 215 220  
 His Thr Glu Ser Gly Lys Thr Cys Gln Arg Trp Asp Gln Gln Thr Pro  
 225 230 235 240  
 His Arg His Lys Phe Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe Asp  
 245 250 255  
 Asp Asn Tyr Cys Arg Asn Pro Asp Gly Lys Pro Arg Pro Trp Cys Tyr  
 260 265 270  
 Thr Leu Asp Pro Asp Thr Pro Trp Glu Tyr Cys Ala Ile Lys Thr Cys  
 275 280 285  
 Ala His Ser Ala Val Asn Glu Thr Asp Val Pro Met Glu Thr Thr Glu  
 290 295 300  
 Cys Ile Gln Gly Gln Gly Glu Gly Tyr Arg Gly Thr Ser Asn Thr Ile  
 305 310 315 320  
 Trp Asn Gly Ile Pro Cys Gln Arg Trp Asp Ser Gln Tyr Pro His Lys  
 325 330 335  
 His Asp Ile Thr Pro Glu Asn Phe Lys Cys Lys Asp Leu Arg Glu Asn  
 340 345 350  
 Tyr Cys Arg Asn Pro Asp Gly Ala Glu Ser Pro Trp Cys Phe Thr Thr  
 355 360 365  
 Asp Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Lys Cys Asp  
 370 375 380

Val Ser Ser Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys Asn Tyr Met  
 385 390 395 400  
 Gly Asn Leu Ser Lys Thr Arg Ser Gly Leu Thr Cys Ser Met Trp Asp  
 405 410 415  
 Lys Asn Met Glu Asp Leu His Arg His Ile Phe Trp Glu Pro Asp Ala  
 420 425 430  
 Ser Lys Leu Asn Lys Asn Tyr Cys Arg Asn Pro Asp Asp Ala His  
 435 440 445  
 Gly Pro Trp Cys Tyr Thr Gly Asn Pro Leu Ile Pro Trp Asp Tyr Cys  
 450 455 460  
 Pro Ile Ser Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val Asn Leu  
 465 470 475 480  
 Asp His Pro Val Ile Ser Cys Ala Lys Thr Lys Gln Leu Arg Val Val  
 485 490 495  
 Asn Gly Ile Pro Thr Gln Thr Thr Val Gly Trp Met Val Ser Leu Lys  
 500 505 510  
 Tyr Arg Asn Lys His Ile Cys Gly Gly Ser Leu Ile Lys Glu Ser Trp  
 515 520 525  
 Val Leu Thr Ala Arg Gln Cys Phe Pro Ala Arg Asn Lys Asp Leu Lys  
 530 535 540  
 Asp Tyr Glu Ala Trp Leu Gly Ile His Asp Val His Glu Arg Gly Glu  
 545 550 555 560  
 Glu Lys Arg Lys Gln Ile Leu Asn Ile Ser Gln Leu Val Tyr Gly Pro  
 565 570 575  
 Glu Gly Ser Asp Leu Val Leu Leu Lys Leu Ala Arg Pro Ala Ile Leu  
 580 585 590  
 Asp Asn Phe Val Ser Thr Ile Asp Leu Pro Ser Tyr Gly Cys Thr Ile  
 595 600 605  
 Pro Glu Lys Thr Thr Cys Ser Ile Tyr Gly Trp Gly Tyr Thr Gly Leu  
 610 615 620  
 Ile Asn Ala Asp Gly Leu Leu Arg Val Ala His Leu Tyr Ile Met Gly  
 625 630 635 640  
 Asn Glu Lys Cys Ser Gln His His Gln Gly Lys Val Thr Leu Asn Glu  
 645 650 655

Ser Glu Leu Cys Ala Gly Ala Glu Lys Ile Gly Ser Gly Pro Cys Glu  
660 665 670

Gly Asp Tyr Gly Gly Pro Leu Ile Cys Glu Gln His Lys Met Arg Met  
675 680 685

Val Leu Gly Val Ile Val Pro Gly Arg Gly Cys Ala Ile Pro Asn Arg  
690 695 700

Pro Gly Ile Phe Val Arg Val Ala Tyr Tyr Ala Lys Trp Ile His Lys  
705 710 715 720

Val Ile Leu Thr Tyr Lys Leu  
725

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cggagagacg cggccgcctc ggcctcgacg ccagcccagg cgccgacctc cgattctcct 180  
gtcgccgagg acgcctcccg gaggcggccg tgccgggcct gcgtcgactt caagacgtgg 240  
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gaactgggcc gccacagctg ggctgtcctc cacaccctgg ccgcctacta ccccgacctg 360  
cccacccag aacagcagcg agacatggcc cagttcatac atttattttc taagttttac 420  
ccctgtgagg agtgtgctga agacctaaga aaaaggttgt gcaggaacca cccagacacc 480  
cgcacccggg catgcttcac acagtggctg tgccacctgc acaatgaagt gaaccgcgag 540  
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gatggctcct gtgactag 618

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<211> 275  
<212> PRT  
<213> Homo sapiens

<400> 14

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Asn Leu Gln His Val Ala Asp Gly Leu Tyr Gly Ser His Leu His Val  
20 25 30

Tyr Ser Trp Pro Gly Gly Glu Ile Lys Gln Leu Ile Asp Leu Gly Pro  
Page 26

35                      40                      45  
 Thr Gly Leu Leu Pro Leu Glu Ile Arg Phe Leu His Asp Pro Ser Lys  
 50                      55                      60  
 Asp Thr Gly Phe Val Gly Ser Ala Leu Ser Ser Asn Met Ile Arg Phe  
 65                      70                      75                      80  
 Phe Lys Asn Ser Asp Glu Thr Trp Ser His Glu Val Val Ile Ser Val  
 85                      90                      95  
 Lys Pro Leu Lys Val Glu Asn Trp Ile Leu Pro Glu Met Pro Gly Leu  
 100                      105                      110  
 Ile Thr Asp Phe Leu Ile Ser Leu Asp Asp Arg Phe Ile Tyr Phe Val  
 115                      120                      125  
 Asn Trp Leu His Gly Asp Ile Arg Gln Tyr Asn Ile Glu Asp Pro Lys  
 130                      135                      140  
 Asn Pro Val Leu Thr Gly Gln Ile Trp Val Gly Gly Leu Leu Gln Lys  
 145                      150                      155                      160  
 Gly Ser Pro Val Lys Ala Val Gly Glu Asp Gly Asn Thr Phe Gln Phe  
 165                      170                      175  
 Glu Val Pro Gln Ile Lys Gly Lys Ser Leu Arg Gly Gly Pro Gln Met  
 180                      185                      190  
 Ile Gln Leu Ser Leu Asp Gly Lys Arg Leu Tyr Ala Thr Asn Ser Leu  
 195                      200                      205  
 Phe Ser Ala Trp Asp Arg Gln Phe Tyr Pro Glu Ile Met Glu Lys Gly  
 210                      215                      220  
 Ser His Ile Ile Gln Ile Asp Val Asp Thr Glu Lys Gly Gly Leu Thr  
 225                      230                      235                      240  
 Ile Asn Pro Asp Phe Phe Val Asp Phe Gly Asp Glu Pro Asp Gly Pro  
 245                      250                      255  
 Ser Leu Ala His Glu Met Arg Tyr Pro Gly Gly Asp Cys Thr Ser Asp  
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 <212> DNA  
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 tacgtggcc gcctattacc cggacaggcc cagccagaa caacaacagg atatggccca 240  
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 tctgagccaa cacttggtt 559

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<400> 16

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Gln Asp Arg Glu Glu Leu Gly Arg His Thr Trp Ala Phe Leu His Thr  
 20 25 30

Leu Ala Ala Tyr Tyr Pro Asp Arg Pro Thr Pro Glu Gln Gln Asp  
 35 40 45

Met Ala Gln Phe Ile His Ile Phe Ser Lys Phe Tyr Pro Cys Glu Glu  
 50 55 60

Cys Ala Glu Asp Ile Arg Lys Arg Ile Gly Arg Asn Gln Pro Asp Thr  
 65 70 75 80

Ser Thr Arg Val Ser Phe Ser Gln Trp Leu Cys Arg Leu His Asn Glu  
 85 90 95

Val Asn Arg Lys Leu Gly Lys Pro Asp Phe Asp Cys Ser Arg Val Asp  
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Glu Arg Trp Arg Asp Gly Trp Lys Asp Gly Ser Cys Asp  
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 <211> 600  
 <212> DNA  
 <213> Mus musculus

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 gctttcctcc atacgtggc cgcctattac ccggacaggc ccacgccaga acaacaacag 360  
 gatatggccc agttcataca tatattttcc aagttttacc cctgcgagga atgtgcggaa 420  
 gacataagga agaggatagg caggaaccag ccagacacaa gcaactcgagt atccttcagc 480  
 cagtggctgt gccgcctgca caatgaggtg aatcggaagc tgggcaagcc tgattttgac 540  
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 <211> 198  
 <212> PRT  
 <213> Mus musculus

<400> 18

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Ser Phe Leu Pro Gly Gly Ala Arg Ser Glu Met Thr Asp Asp Leu Val  
 20 25 30

Thr Asp Ala Arg Gly Arg Gly Ala Arg His Arg Asp Asp Thr Thr Pro  
 35 40 45

Ser Ala Ala Pro Ala Pro Gln Gly Leu Glu His Gly Lys Arg Pro Cys  
 50 55 60

Arg Ala Cys Val Asp Phe Lys Ser Trp Met Arg Thr Gln Gln Lys Arg  
 65 70 75 80

Asp Ile Lys Phe Arg Glu Asp Cys Pro Gln Asp Arg Glu Glu Leu Gly  
 85 90 95

Arg His Thr Trp Ala Phe Leu His Thr Leu Ala Ala Tyr Tyr Pro Asp  
 100 105 110

Arg Pro Thr Pro Glu Gln Gln Gln Asp Met Ala Gln Phe Ile His Ile  
 115 120 125

Phe Ser Lys Phe Tyr Pro Cys Glu Glu Cys Ala Glu Asp Ile Arg Lys  
 130 135 140

Arg Ile Gly Arg Asn Gln Pro Asp Thr Ser Thr Arg Val Ser Phe Ser  
 145 150 155 160

Gln Trp Leu Cys Arg Leu His Asn Glu Val Asn Arg Lys Leu Gly Lys  
 165 170 175

Pro Asp Phe Asp Cys Ser Arg Val Asp Glu Arg Trp Arg Asp Gly Trp  
 180 185 190

Lys Asp Gly Ser Cys Asp  
 195

<210> 19  
 <211> 1869  
 <212> DNA  
 <213> Homo sapiens

<400> 19  
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 ctataggcag ctgctcgaaa gagtaccgcg tgctccttgg ccagctccag aagcagacag 180  
 atctcatgca ggacaccagc agactcctgg acccctatat acgtatccaa ggcctggatg 240  
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 tgagggggct gggcaggcgg ggcttcctgc agaccctcaa tgccacactg ggctgcgtcc 360  
 tgcacagact ggccgactta gagcagcgcc tccccaaggc ccaggatttg gagaggctctg 420  
 ggctgaacat cgaggacttg gagaagctgc agatggcgag gccgaacatc ctcgggctca 480  
 ggaacaacat ctactgcatg gcccagctgc tggacaactc agacacggct gagccacga 540  
 aggctggccg gggggcctct cagccgcca cccccaccc tgctcggat gcttttcagc 600  
 gcaagctgga gggctgcagg ttctgcatg gctaccatcg cttcatgcac tcagtggggc 660  
 gggctttcag caagtggggg gagagcccga accggagccg gagacacagc cccaccagg 720  
 ccctgaggaa ggggggtgcg aggaccagac cctccaggaa aggcaagaga ctcatgacca 780  
 ggggacagct gccccggtag cctcgagagc accccttgcc ggtgaaggat gcggcagggtg 840  
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 ctctgctact agccactgag aagtgcactt taagagggtg gagctgggca gaccctcta 960  
 cctcctccag gctgggagac agagtcaggc tgttgcgctc ccacctcagc cccaagttcc 1020  
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 tctgatgaca caggctgact catggccggg ctgactgccc ccctgccttg ctccccgagg 1140  
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 gtgtttctga aggggaggtc acagcctgag ctggcctcct atgcctcatc atgtcccaaa 1260  
 ccagacacct ggatgtctg gtgacctcac tttaggcagc tgtaacagcg gcagggtgtc 1320  
 ccaggagccc tgatccgggg gtccagggaa tggagctcag gtcccaggcc agccccgaag 1380  
 tcgccacgtg gcctggggca ggtcacttta cctctgtgga cctgttttct ctttgtgaag 1440



ctagggagtt agaggctgta caaggccccc actgcctgtc ggttgcttgg attccctgac 1500  
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 gtacacggag gtctctgtaa aagcagaccc acctcccagc gccgggaagc ccgtcttggg 1620  
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 ttgctctcct agaggggggtc tcagaaactg cgaggccagt tccttggagg gacatgacta 1740  
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 aaaaaaaaaa 1869

<210> 20  
 <211> 252  
 <212> PRT  
 <213> Homo sapiens  
 <400> 20

Met Gly Val Leu Leu Thr Gln Arg Thr Leu Leu Ser Leu Val Leu Ala  
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Leu Leu Phe Pro Ser Met Ala Ser Met Ala Ala Ile Gly Ser Cys Ser  
 20 25 30

Lys Glu Tyr Arg Val Leu Leu Gly Gln Leu Gln Lys Gln Thr Asp Leu  
 35 40 45

Met Gln Asp Thr Ser Arg Leu Leu Asp Pro Tyr Ile Arg Ile Gln Gly  
 50 55 60

Leu Asp Val Pro Lys Leu Arg Glu His Cys Arg Glu Arg Pro Gly Ala  
 65 70 75 80

Phe Pro Ser Glu Glu Thr Leu Arg Gly Leu Gly Arg Arg Gly Phe Leu  
 85 90 95

Gln Thr Leu Asn Ala Thr Leu Gly Cys Val Leu His Arg Leu Ala Asp  
 100 105 110

Leu Glu Gln Arg Leu Pro Lys Ala Gln Asp Leu Glu Arg Ser Gly Leu  
 115 120 125

Asn Ile Glu Asp Leu Glu Lys Leu Gln Met Ala Arg Pro Asn Ile Leu  
 130 135 140

Gly Leu Arg Asn Asn Ile Tyr Cys Met Ala Gln Leu Leu Asp Asn Ser  
 145 150 155 160

Asp Thr Ala Glu Pro Thr Lys Ala Gly Arg Gly Ala Ser Gln Pro Pro  
 165 170 175

Thr Pro Thr Pro Ala Ser Asp Ala Phe Gln Arg Lys Leu Glu Gly Cys  
180 185 190

Arg Phe Leu His Gly Tyr His Arg Phe Met His Ser Val Gly Arg Val  
195 200 205

Phe Ser Lys Trp Gly Glu Ser Pro Asn Arg Ser Arg Arg His Ser Pro  
210 215 220

His Gln Ala Leu Arg Lys Gly Val Arg Arg Thr Arg Pro Ser Arg Lys  
225 230 235 240

Gly Lys Arg Leu Met Thr Arg Gly Gln Leu Pro Arg  
245 250

<210> 21  
<211> 1848  
<212> DNA  
<213> Mus musculus

<400> 21  
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gctgctcaa ctcttctct cagtcctca gccagctgca gaatcaggcg aacctcacgg 180  
ggaacacaga atcactcttg gagccctata tccgcctcca aaacctgaac acactgacc 240  
tgagagctgc ctgcaccag cactctgtgg ctttccccag tgaggacaca ctccggcaac 300  
tgagcaagcc tcaacttctg agcactgtgt acaccacact ggacagagtc ttgtaccaac 360  
tggatgcttt aagacagaaa tttctgaaga ctccggcttt tccaaagctg gacagtgcc 420  
ggcacaatat cctcggcata aggaacaatg ttttctgcat ggcccggctg ctcaaccact 480  
ccctggagat acctgagccc acacagacag actctggggc ctacagggtcc actacaacac 540  
cagatgtctt taataccaag ataggcagct gtggctttct ctggggatac catcgcttca 600  
tgggctcagt ggggagggc ttcagggaat gggacgatgg ctccacacgc agccggagac 660  
agagcccgtc ccgggcccgg cgcaagggaa cccgcagaat ccgggtccgg cacaagggaa 720  
cccgcagaat ccgggtccgg cgcaagggaa cccgcagaat ctgggtccgg cgcaagggat 780  
cccgcaaat cagaccttcc aggagcacc agagcccag gaccagggcc taggttccct 840  
ggtagcctga ggacacactg acagacagca tagtctggtg atacaggatg tcgctctcag 900  
aggctttcaa agctgcttct gtcaccagg gtcacacaga agagcacttt aaggggtgaa 960  
gttgagtgtc ccctactacc actcaggact tcaaggatag tgaggttatt gtgtccccac 1020  
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ctcgggctgt tggctcttcc ctctcatgac ttgaaactgt ttctccaga ctctctctt 1200

tccctgtggc tgggttccaa agagaggtct gatccggtgc tctcttcat gccttatccc 1260  
 actcaggaca gatacctgga cctctgggtg acctcacact tggcagttgc gacaggggca 1320  
 ggggtgccta ccaaggaaca ctgatctggg cgttcaggga agagagctca gagcctagct 1380  
 tgttccctaa ttctctgtgt gactgtgagc aagacacttt atttatccga atgtcagcgt 1440  
 tctctgtgga aagctgtgtt gtgtgtgtgg gtccttaggt aatagccccc tctgcctgtc 1500  
 agctgctgga ctctgacata ggggtggacat caaagtctct gtaaattggga acctgtgggtg 1560  
 caaacggttg tgggggtgtgt ttatgggaga tctcccagtg cctaaaagcc ctgttttggg 1620  
 tcctcgctgc atgatgtccc ctctgggtgat gtgtgtgaa atttttcaca ggctgaacca 1680  
 gtcctcttga aaggtctcag aagctgggtga gcaattactt ggagggacat gactaattta 1740  
 ttgttttatt ttttatcagt ttaatccgtt ttatatattat aaggcctatt tataatgtat 1800  
 atttaatggt aatattttgc taacatattt aaaacctgtc ttgtttct 1848

<210> 22  
 <211> 263  
 <212> PRT  
 <213> Mus musculus

<400> 22

Met Gln Thr Arg Leu Leu Arg Thr Leu Leu Ser Leu Thr Leu Ser Leu  
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Leu Ile Leu Ser Met Ala Leu Ala Asn Arg Gly Cys Ser Asn Ser Ser  
20 25 30

Ser Gln Leu Leu Ser Gln Leu Gln Asn Gln Ala Asn Leu Thr Gly Asn  
35 40 45

Thr Glu Ser Leu Leu Glu Pro Tyr Ile Arg Leu Gln Asn Leu Asn Thr  
50 55 60

Pro Asp Leu Arg Ala Ala Cys Thr Gln His Ser Val Ala Phe Pro Ser  
65 70 75 80

Glu Asp Thr Leu Arg Gln Leu Ser Lys Pro His Phe Leu Ser Thr Val  
85 90 95

Tyr Thr Thr Leu Asp Arg Val Leu Tyr Gln Leu Asp Ala Leu Arg Gln  
100 105 110

Lys Phe Leu Lys Thr Pro Ala Phe Pro Lys Leu Asp Ser Ala Arg His  
115 120 125

Asn Ile Leu Gly Ile Arg Asn Asn Val Phe Cys Met Ala Arg Leu Leu  
130 135 140

Asn His Ser Leu Glu Ile Pro Glu Pro Thr Gln Thr Asp Ser Gly Ala  
145 150 155 160

Ser Arg Ser Thr Thr Thr Pro Asp Val Phe Asn Thr Lys Ile Gly Ser  
165 170 175

Cys Gly Phe Leu Trp Gly Tyr His Arg Phe Met Gly Ser Val Gly Arg  
180 185 190

Val Phe Arg Glu Trp Asp Asp Gly Ser Thr Arg Ser Arg Arg Gln Ser  
195 200 205

Pro Leu Arg Ala Arg Arg Lys Gly Thr Arg Arg Ile Arg Val Arg His  
210 215 220

Lys Gly Thr Arg Arg Ile Arg Val Arg Arg Lys Gly Thr Arg Arg Ile  
225 230 235 240

Trp Val Arg Arg Lys Gly Ser Arg Lys Ile Arg Pro Ser Arg Ser Thr  
245 250 255

Gln Ser Pro Thr Thr Arg Ala  
260

<210> 23  
<211> 33  
<212> DNA  
<213> Mus musculus

<400> 23  
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33

<210> 24  
<211> 31  
<212> DNA  
<213> Mus musculus

<400> 24  
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31

<210> 25  
<211> 32  
<212> DNA  
<213> Mus musculus

<400> 25  
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32

<210> 26  
<211> 424  
<212> DNA  
<213> Mus musculus

<400> 26  
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60

gaagaattgg gtcgccacac ctgggctttc ctccatacgc tggccgccta ttacccggac 120  
 agggccacgc cagaacaaca acaggatatg gccagttca tacatatatt ttccaagttt 180  
 tacccttgcg aggaatgtgc ggaagacaça aggaagagga taggcaggaa ccagccagac 240  
 acaagcactc gagtatcctt cagccagtgg ctgtgccgcc tgcacaatga ggtgaatcgg 300  
 aagctgggca agcctgattt tgactgctcg agagtagatg agcgttggcg tgacggatgg 360  
 aaggacggct cctgtgacta gtgaaagggc gaattctgca gatatccatc aactggcgg 420  
 ccgc 424

<210> 27  
 <211> 22  
 <212> DNA  
 <213> Mus musculus

<400> 27  
 cagccaaagt ggagtggaaa ga 22

<210> 28  
 <211> 21  
 <212> DNA  
 <213> Mus musculus

<400> 28  
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<210> 29  
 <211> 22  
 <212> DNA  
 <213> Mus musculus

<400> 29  
 attgagaaga cccctgcctt gt 22

<210> 30  
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<400> 30  
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<400> 33  
cctgaaccaa tcccacctct ct 22

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<400> 34  
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attgagaaga cccctgcctt gt 22

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<400> 36  
atctgcaatg tgtagccag c 21

<210> 37  
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<213> Mus musculus

<400> 37  
aagaagatgg ctttcaggcc c 21

<210> 38  
<211> 21  
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<400> 38  
aaggccattg aagtgtggtg g 21

<210> 39  
<211> 22  
<212> DNA  
<213> Mus musculus

<400> 39  
gactctctaa aacccttgcc gg 22

<210> 40  
<211> 21

<212> DNA  
<213> Mus musculus  
<400> 40  
ccatggtcaa cacctgcaca t 21

<210> 41  
<211> 22  
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<400> 41  
cgcccatcgg tataatgatt tg 22

<210> 42  
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<400> 42  
ctgcactaat ttggcatgct ca 22

<210> 43  
<211> 20  
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<213> Mus musculus  
<400> 43  
tgcttgatgt gcaccattgc 20

<210> 44  
<211> 21  
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<213> Mus musculus  
<400> 44  
tgctccagat gctgcatctt c 21

<210> 45  
<211> 21  
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<400> 45  
aagcagagtg gaccaaccgt t 21

<210> 46  
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<213> Mus musculus  
<400> 46  
aagcagagtg gaccaaccgt t 21

<210> 47  
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<400> 47

ttaatgtgct tggcccgatc

20

<210> 48

<211> 22

<212> DNA

<213> Mus musculus

<400> 48

ccagcgaagg cttgttttag aa

22